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Pre-clinical characterisation of mitochondrial antioxidants as novel therapeutics for Pre-eclampsia.

Thesis presented by

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In fulfilment of the requirements for the degree of

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Under the supervision of

Dr Cathal McCarthy, Dr Fergus McCarthy and Prof Louise Kenny

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Contents

Declaration

This thesis comprises original work carried out by the author (unless otherwise stated) and has not been submitted in whole or in part for any other degree, diploma or qualification at any other university.

Author Contribution

All of the work described herein was performed independently by the author, with the following exceptions:

Chapter 2:

Ainhoa Totorika assisted with the Superoxide Dismutase experiments.

Chapter 4:

Robert Kerley assisted with the cell viability and dose response experiments in the placental cell culture experiments.

Chapter 5:

Cayman Chemical assisted with mitochondrial hydrogen peroxide determination in rat kidneys.

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all been there to see me through, none of this could have been achieved without you all.

Xx

Abbreviations

9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin F2α	U46619
Adenosine triphosphate	ATP
Alanine transaminase	ALT
Alkoxy-Radical	LO
American College of Obstetrics and Gynaecology	ACOG
Asymmetrical dimethylarginne	ADMA
Body mass index	BMI
Bradykinin	BK
Cell-free DNA	cf-DNA
Damage-associated molecular patterns	DAMPs
DNA dependent protein-kinase	DNAPKcs
Electron transport chain	ETC
Endogenous Nitric oxide synthesis	eNOS
Endoglin	Eng
Enzyme-linked immunosorbent assay	ELISA
Fetal growth restriction	FGR
Flavin adenine dinucleotide	FADH2
Glutathione peroxidase	GPx
Guanosine triphosphatases	GTPases

Hemolysis, Elevated Liver enzymes and Low Platelets	HELLP
Human chorionic gonadotropin	hCG
Human placental lactogen	hPL
Human umbilical vein endothelial cell	HUVEC
Hydrogen Peroxide	H_2O_2
Hydroxyl radical	HO
Hypochlorous acid	$HOCl^-$
Interferon gamma	IFN-γ
Interleukin 1	IL-1
Interleukin 1 beta	IL-1β
Interleukin 10	IL-10
Interleukin 13	IL-13
Interleukin 2	IL-2
Interleukin 4	IL-4
Interleukin 5	IL-5
Interleukin 6	IL-6
Interleukin 8	IL-8
Interleukin-1 receptor-associated kinase	IRAK
Intrauterine growth restriction	IUGR
L-Ergothioneine	ERG

Lipid Peroxy-radicals	LOO
Malondialdehyde	MDA
Manganese superoxide dismutase	mnSOD
Matrix Metalloproteinase	MMPs
Matrix Metalloproteinase-8	MMP-8
Mean Arterial Blood Pressure	MABP
Mitochondrial Damage-associated molecular patterns	mtDAMPs
mitochondrial DNA	mtDNA
Mitochondrial reactive oxygen species	mROS
Mitogen-activated kinase	MAPK
Myeloperoxidase	MPO
Neutrophil extracellular traps	NETs
Nicotinamide adenine dinucleotide	NADH
Nicotinamide adenine dinucleotide phosphate	NADPH
Nitic Oxide	NO
Nitric Oxide synthesis	NOS
Nuclear factor erythroid 2-related factor 2	Nrf2
Organic cation transporter novel type 1	OCTN1
Oxidative stress	OS
Pathogen associated molecular patterns	PAMPs

Peroxisome proliferator-activated receptor gamma coactivator alpha	PCG-1α
Peroxynitrite	ONOO-
Physiological salt solution	PSS
Placental growth factor	PlGF
Potassium physiological salt solution	KPSS
Proton motive force	pmf
Randomised Clinical trial	RCT
Reactive Nitrogen Species	RNS
Reactive Oxygen Species	ROS
Reduced uterine perfusion pressure model	RUPP
Royal College Of Obstetrics and Gynaecology	RCOG
Screening for Pregnancy Endpoints study	SCOPE
Selenium atom	Se
Small for gestational age	SGA
Sodium Nitropusside	SNP
Soluble Endoglin	sEng
Soluble fms-like tyrosine kinase 1	sFlt-1
Superoxide	O_2^-
Superoxide Dismutase	SOD
The International Society for the Study of Hypertension in Pregnancy	ISSHP

T-helper	Th
Thiordeoxin Reductase	ThxRed
Time of Disease	TOD
Toll-like receptors	TLRs
Triphenylphosphonium	TTP
Tumour necrosis factor alpha	TNF-α
Uncoupling protein-1	UCP-1
Vascular endothelial growth factor	VEGF
Vitamin C and vitamin E in pregnant women at risk for pre-eclampsia	VIP trial
Xanthine dehydrogenase	XDH

Dissemination of work

Publications

Published manuscripts

Williamson RD, Cathal McCarthy, Fergus McCarthy, Louise C. Kenny. Oxidative stress in pre-eclampsia; have we been looking in the wrong place? Pregnancy Hypertension 2017. DOI: http://dx.doi.org/10.1016/j.preghy.2017.01.004.

Williamson RD, Fergus P McCarthy, Ali S. Khashan, Ainhoa Totorika, Louise C Kenny, Cathal McCarthy. Exploring the role of mitochondrial dysfunction in the pathophysiology of pre-eclampsia. Pregnancy Hypertension 2018. https://doi.org/10.1016/j.preghy.2018.06.012.

Williamson RD, Fergus P McCarthy, Louise C Kenny, Cathal McCarthy. Activation of a TLR9 mediated innate immune response in pre-eclampsia. Scientific Reports 2019. https://doi.org/10.1038/s41598-019-42551-w

Manuscripts Accepted for publication

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Manuscripts Submitted for publication

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- College of Medicine and Health Travel Bursary in 2017 to disseminate my research in Berlin at the European Society for the Study of Hypertension in pregnancy

Papers associated with chapters in this thesis

Chapter 1

Williamson RD, Cathal McCarthy, Fergus McCarthy, Louise C. Kenny. Oxidative stress in pre-eclampsia; have we been looking in the wrong place? Pregnancy Hypertension 2017. DOI: http://dx.doi.org/10.1016/j.preghy.2017.01.004.

Chapter 2

Williamson RD, Fergus P McCarthy, Ali S. Khashan, Ainhoa Totorika, Louise C Kenny, Cathal McCarthy. Exploring the role of mitochondrial dysfunction in the pathophysiology of pre-eclampsia. Pregnancy Hypertension 2018. https://doi.org/10.1016/j.preghy.2018.06.012.

Chapter 3

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Manuscripts Accepted

Chapter 5

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Abstract

Pre-eclampsia, a pregnancy specific condition associated with significant morbidity and mortality, is characterised by high blood pressure accompanied by and/or proteinuria, acute kidney injury (AKI), liver dysfunction fetal growth restriction (FGR) after 20 weeks' gestation in the second half of pregnancy. It affects 2-5% of pregnancies worldwide. There is overwhelming evidence showing oxidative stress plays a significant role in the aetiology of this disorder. The mitochondria are known to be a dominant cellular producer of reactive oxygen species. Mitochondrial dysfunction acts as a pathogenic mediator of oxidative stress in pre-eclampsia and modulates the clinical characteristics of this syndrome. There is evidence that mitochondrial dysfunction and the innate immune system both play roles in the pathophysiology of this disease. Mitochondrial damage-associated molecular patterns (DAMPs) such as mtDNA bind specific pattern recognition receptors such as Toll-like receptor 9 (TLR9) on the surface of immune cells, particularly neutrophils, subsequently activating them and triggering an innate immune response.

Currently there is no treatment for pre-eclampsia except delivery of the placenta and the baby, with the resultant risk of iatrogenic prematurity and significant neonatal morbidity and mortality. As a result, intensive research endeavours have focused on defining the molecular mechanisms of pre-eclampsia and thereby expediting identification of new therapeutic targets for this condition. In pregnancies affected by pre-eclampsia there is an exaggerated oxidative stress phenotype with a correspondent deficient antioxidant response. The therapeutic potential of vitamin antioxidants in the prevention of pre-eclampsia was supported by extensive research, however, ultimately, they were unsuccessful in clinical trials. The ongoing hypothesis is that these vitamin antioxidants were sequestered in the cytosol and

didn’t reach the mitochondria to alleviate the excess oxidative stress. Therefore, there is a need for the development of a specific mitochondrial-targeted antioxidant as a potential therapy for pre-eclampsia.

This body of research provided evidence of mitochondrial dysfunction and a deficient antioxidant response in women who developed pre-eclampsia as early as 15 weeks’ gestation. However, there was no association found between lifestyle factors on mitochondrial dysfunction in the study cohort. Furthermore, there was increased activation of TLR9, a mediator of mtDNA induced innate inflammation in the same cohort, albeit at a later point in gestation. Additionally, there was a significant increase in the production of a number of neutrophil activation markers; calprotectin, myeloperoxidase and Interleukin-8 in women who developed pre-eclampsia. In the next study, I examined if L-ergothioneine, a water-soluble amino acid, and potential mitochondrial targeted antioxidant could mediate oxidative stress in an *in vitro* Jeg-3 placental cell line. In this novel study, L-ergothioneine exhibited cytoprotective effects against H_2O_2-induce cell death and significantly reduced exaggerated mitochondrial-specific ROS production in part by altering mild uncoupling. The protective effects of pre-treatment with L-ergothioneine were more pronounced when compared with another mitochondrial targeted antioxidant, MitoTempo and the non-mitochondrial targeted antioxidant N-acetylcysteine.

The final study examined the potential therapeutic effects of L-ergothioneine *in vivo* using the reduced uterine perfusion pressure (RUPP) rat model of pre-eclampsia. In this study, treatment with L-ergothioneine significantly reduced blood pressure and rescued fetal growth restriction in RUPP rats. Furthermore, sFlt-1, an anti-angiogenic factor evident in pre-eclampsia, was significantly decreased in rats treated with L-ergothioneine. Additionally, using a novel ratiometric mass

spectrometry probe, I showed that mitochondrial H_2O_2 production *in vivo* was significantly increased in the kidney from the RUPP rats and that pre-treatment with L-ergothioneine significantly reduced mitochondrial H_2O_2 production *in vivo*.

In summary, this thesis has provided evidence of mitochondrial dysfunction in women who develop pre-eclampsia. Additionally, this work has shown that mitochondrial dysfunction activates a neutrophil-driven innate immune response in women with pre-eclampsia which is mediated in part by activation of the TLR9 receptor. Furthermore, this novel work established that L-ergothioneine, a unique antioxidant directly targets the mitochondria and reduces exaggerated mitochondrial specific ROS production in both *in vitro* and *in vivo* models of pre-eclampsia. Additionally, in the *in vivo* model of pre-eclampsia, treatment with L-ergothioneine positively modulates some of the detrimental clinical characteristics evident in women with pre-eclampsia, highlighting the therapeutic potential of this mitochondrial-targeted antioxidant in the treatment of pre-eclampsia.

Chapter 1: General Introduction

Rachel D Williamson, Cathal M McCarthy, Fergus P McCarthy, Louise C. Kenny. Oxidative stress in pre-eclampsia; have we been looking in the wrong place? Pregnancy Hypertension 2017. DOI: http://dx.doi.org/10.1016/j.preghy.2017.01.004

1.0 Chapter 1: Introduction

1.1.1 Definition and epidemiology of pre-eclampsia

Pre-eclampsia is defined by the International Society for the Study of Hypertension in Pregnancy (ISSHP) as gestational maternal hypertension of at least 140/90mmHg after 20 weeks' gestation and accompanied by proteinuria and/or evidence of maternal acute kidney injury (AKI), neurological features, haemolysis, liver dysfunction, or fetal growth restriction (1). Pre-eclampsia is a major cause of maternal and perinatal mortality and morbidity worldwide, accounting for nearly 18% of all maternal deaths worldwide; an estimated 77,000 maternal deaths per year and affects 10 million women globally (2). This hypertensive pregnancy complication occurs during the second half of pregnancy and complicates around 2-8% of pregnancies (3). Whilst low to middle income countries bear the burden of maternal and perinatal mortality, worryingly, maternal mortality is increasing in some high resource settings due to global epidemics of obesity and diabetes mellitus. When women present with early-onset pre-eclampsia prior to 33 weeks' gestation, the risk of serious maternal and perinatal morbidity and mortality is significantly increased. Maternal and perinatal outcomes are less severe when late-onset pre-eclampsia develops after 36 weeks' gestation. Suspected pre-eclampsia is the most frequent clinical presentation to obstetric day-care units in Ireland and those with early-onset are at greater risk. Preterm delivery is a serious complication of early-onset pre-eclampsia with fetal morbidity and mortality being directly correlated with gestational age at delivery. Furthermore, according to the Perinatal mortality in Ireland report, pregnancies affected by pre-eclampsia had an increased risk of perinatal mortality with 2.9% affected mothers experiencing perinatal loss

(NPECPerinatalMortalityinIrelandAnnualReport2016).

1.1.2 Risk factors and long-term prognosis

The risk of pre-eclampsia is increased in women with a previous history of pre-eclampsia with a relative risk (RR) of 7.19 (95% confidence interval 5.85-8.83). Further risk factors for pre-eclampsia include maternal age, greater than 40 years of age (RR 1.96, 1.34-2.87), pre-existing diabetes (RR 3.56, 2.54-4.99), family history of pre-eclampsia (RR 2.90, 1.70-4.93), raised blood pressure, ≥80 mmHg, at booking (RR 1.38, 1.01-1.87), multiple (twin) pregnancy (RR 2.93, 2.04-4.21) and pre-pregnancy obesity (RR 2.47, 1.66-3.67) (4). Furthermore, pre-eclampsia risk is higher among African American women (5.2%; odds ratio [OR] 1.41, 95% confidence interval [CI] 1.25–1.62) while lower among Latina (4.0%; OR 0.90, 95% CI 0.84–0.97) and Asian women (3.5%; OR 0.79, 95% CI 0.72–0.88), compared with Caucasian women (5).

Short term maternal and fetal complications associated with pre-eclampsia include an increased risk of eclampsia (seizures in the mother). Furthermore, fetal complications include small for gestational age (SGA) and intrauterine growth restriction (IUGR) and in some cases, fetal death (6, 7). Women who develop pre-eclampsia have a further increased risk of developing HELLP (Hemolysis, Elevated Liver enzymes and Low Platelets) syndrome. HELLP syndrome is a complication of severe pre-eclampsia, which can cause liver failure and maternal death, occurs in about 12% of pregnancies complicated by pre-eclampsia or eclampsia (8). Pre-eclampsia has been shown to carry long-term health implications later in adult life for both mother and baby, with offspring of affected pregnancies demonstrating an increased risk of hypertension (9), stroke (10) and diabetes (11). Following a pre-

eclamptic pregnancy, a woman is at an increased risk of developing cardiovascular complications (4-10 fold) (12), with 20% of women developing cardiovascular disease within 7 years (13).

1.1.3 The Screening for Pregnancy Endpoints (SCOPE) International Cohort Study.

More than half of all cases of pre-eclampsia occur in healthy first-time pregnant women. The primary objective of the SCOPE study was to develop a multivariable predictive model for the prediction of preeclampsia by combining measurements of the best performing biomarkers (selected from >100 previously associated with pre-eclampsia) measured at 14 to 16 weeks' gestation, clinical risk factors and ultrasound estimates of uteroplacental perfusion and fetal measurements at 19 to 21 weeks' gestation. This study was carried out across 4 countries including New Zealand, Australia, United Kingdom; and Ireland where 5690 women were recruited.

Pregnancy outcome data were available for 5623 (99%). Pre-eclampsia developed in 278 (4.9%), of which 209 had term pre-eclampsia (3.7%), 69 (1.2%) had preterm pre-eclampsia, and 28 (0.5%) had early-onset pre-eclampsia. The diagnosis of pre-eclampsia was based on hypertension with proteinuria in 90% and on hypertension in combination with multisystem complications in 10% (n=29) (14). A total of 1,774 participants were involved in the Cork cohort, of which 68 (3.8%) women were diagnosed with pre-eclampsia. This multicentre cohort has created a vast amount of publications across all areas of research in regards to maternal and fetal health (14-18)

1.2 Aetiology of pre-eclampsia

1.2.1 Placental development in normal pregnancy

The placenta is a specialised organ of pregnancy along with the fetal membranes and amniotic fluid, which supports the normal growth and development of the fetus. During pregnancy, the placenta plays a number of key roles and functions predominantly at the site of maternal-fetal exchange. These include; 1) exchange of oxygen and CO_2; 2) as a digestive system which supplies nutrients for fetal growth and development; 3) removes waste products; 4) as an immune barrier that prevents any antigenic attacks from the mother; 5) as a key mediator of endocrine function during pregnancy. From the earliest days of pregnancy, trophoblasts generate a variety of hormones including gonadotropin and progesterone. The primary source of these placental hormones is the syncytiotrophoblast layer. The two most important protein hormones produced by the placenta are human chorionic gonadotropin (hCG) and human placental lactogen (hPL) (19, 20).

hCG is involved in pleotropic roles throughout pregnancy owing to its autocrine and paracrine actions, in turn linking with many processes that are crucial for the pregnancy outcome. In pregnancy this glycoprotein is critical since it rescues the corpus luteum from involution, which subsequently maintains progesterone secretion by the ovarian granulosa cells. Similarly, progesterone is crucial for gestational maintenance. After 6-8 weeks of gestation, hCG peaks and tends to plateau to a lower level for the remainder of pregnancy. Therefore, the production of progesterone declines due to diminished placenta production of hCG and the placental trophoblasts take over as the main source of progesterone production (21). Human placental lactogen (hPL), is produced and secreted by the syncytiotrophoblast layer of the placenta and is first detected in the maternal circulation at 6 weeks of gestation (22). This hormone modifies the metabolic state of the mother during pregnancy to facilitate the energy supply of the fetus.

Placental development involves the coordination and function of many cell types including cytotrophoblasts and syncytiotrophoblasts (23). During the second week of development, implantation of the blastocyst in the endometrium of the uterus is complete. The amniotic cavity, yolk sac, connecting stalk and chorion equally develop during the second week. Villi are first recognisable around the 12^{th} day of development. During normal implantation, the uterine spiral arterioles undergo extensive remodelling as they are invaded by endovascular trophoblasts. Endovascular trophoblasts invade and replace the vascular endothelial and muscular linings to enlarge the vessel diameter (24, 25). Maternal spiral arterioles are eroded on the 14^{th} or 15^{th} day, and maternal arterial blood enters the developing intervillous space. Both fetal and maternal blood vessels are active and the placental circulation is established around the 17^{th} day after conception (19). At the time of implantation, the actively erosive trophoblasts continue to invade the endometrium containing connective tissues, capillaries and glands in a process called placentation. As trophoblasts invade the endometrium, they differentiate into two distinct layers; cytotrophoblast and syncytiotrophoblast.

The cytotrophoblast is composed of mononuclear cells whereas the syncytiotrophoblast consists of a thick multinucleated protoplasmic mass. There are spaces left between syncytial cells which are known as the lacunae and they appear in the syncytiotrophoblast and soon become filled with blood from ruptured maternal capillaries. The successful development of a normal placenta is dependent on the orchestrated balance of cytotrophoblast cell proliferation and differentiation into either one of the differentiated cell types, invasive cytotrophoblasts or syncytiotrophoblast (26). After implantation, the invasive trophoblasts proliferate and differentiate along two pathways defined as villous and extravillous. Villous

cytotrophoblast cells fuse to form the multinucleated syncytiotrophoblast, whereas the extravillous trophoblasts form the outer epithelial later of the chorionic villi. Differentiating and invading cytotrophoblasts undergo a process known as pseudo-vasculogenesis, where these cells acquire an adhesion receptor repertoire resembling that of endothelial cells (27). There are a variety of molecular families including vascular endothelial growth factor (VEGF) and placental growth factor (PLGF) that play important roles in vasculogenesis and/or angiogenesis (28). Both maternal and fetal macrophages in addition to cytotrophoblasts produce these angiogenic mediators in the placenta (29, 30).

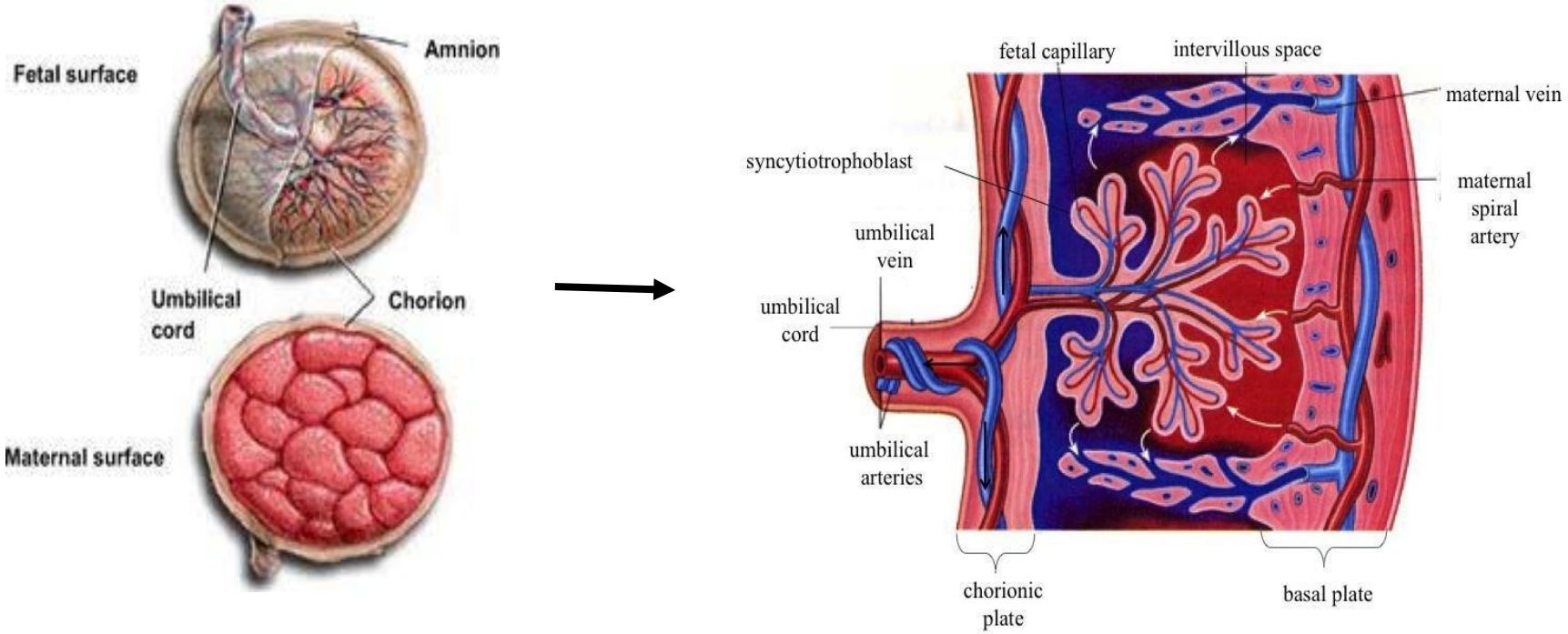

Figure 1.1: Placental structure: The placenta has two surfaces, maternal and fetal. The chorion, amnion, yolk sac and allantois constitute the fetal membranes. The appearance of the maternal surface of a placenta is a "cobblestone–like" structure consisting of 10-38 cotyledons. A cotyledon is made up of approximately 10 and 20 lobules which then correspond with the opening of a maternal uteroplacental vessel. Illustration was sourced from Lofthouse et al (31).

1.2.2 Placental development in pre-eclampsia

In pre-eclamptic pregnancies placentation is compromised and incomplete, where remodelling of spiral arterioles and the uterine decidua and myometrium is inadequate which in turn leads to shallow extravillous trophoblast (EVT) invasion and migration (Figure 1.2). In pre-eclampsia, there is histopathological evidence of incomplete trophoblastic invasion. Pathological changes are the most common with severe pre-eclampsia (32). In a large study cohort (n = 6410) a series of placental measurements were performed in Finland between 1934 and 1944 (33). They found that rather than the circular shape usually present in pregnancy, there was a greater discrepancy between the shortest and longest diameter in placentas from pre-eclampsia pregnancies. The hypothesis is that such an abnormal placental shape is associated with reduced endovascular invasion (34). As a result only the decidual vessels become lined with endovascular trophoblasts while the myometrial vessels remain uninvaded and narrow (25). Histologically, normal third-trimester decidual vessels are characterized by flat endothelium and a loss of medial smooth muscle, while pre-eclamptic decidua show signs of loose, oedematous endothelium, hypertrophy of the vessel media, and loss of smooth muscle modifications (35). In more recent years, atherosclerotic changes in maternal radial arteries that supply the decidua are examined as opposed to the spiral arteries and have been observed in pre-eclampsia. Therefore, due to deficient trophoblast invasion and narrow vessel structure there is an intermittency of arterial O_2 blood flow delivered to the developing placenta and fetus, creating a hypoxic/ischaemic environment (36).

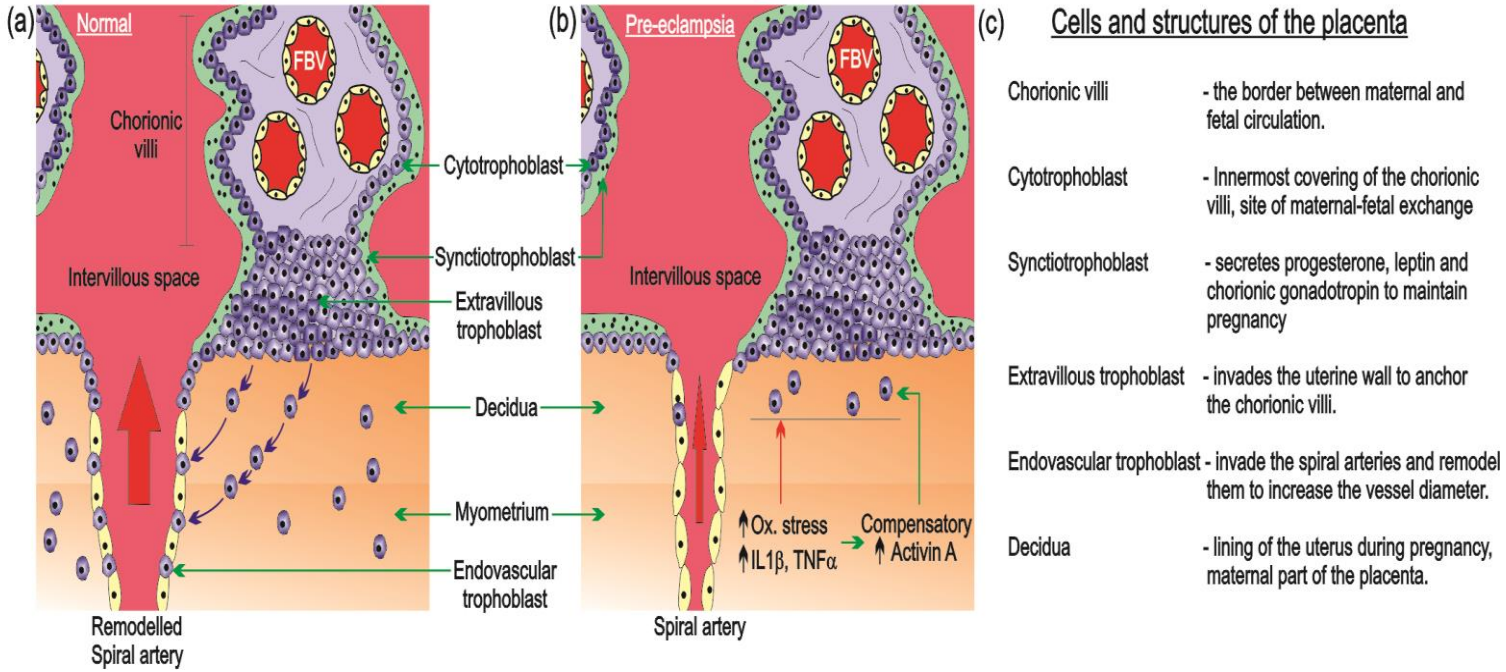

Figure 1.2: Remodelling of the spiral arteries by the trophoblast.

(a) During implantation, trophoblastic cells (shown in blue) migrate from the anchoring villi and invade the vascular endothelium and muscular layers of the uterine spiral arterioles (shown in yellow), where they enlarge the vessel diameter and create a high capacitance, low resistance circulation, a process known as “remodelling”. (b) However, in pre-eclampsia, this trophoblastic invasion is incomplete. This has been proposed to be due to increased oxidative stress and cytokine production which impairs migration and invasion of the

extravillous trophoblast. (c) List of major placental structures and the various trophoblast cell types associated with them. This figure was created by Williamson et al (37).

Several years ago a concept originally introduced by Professor Chris Redman and colleagues described how pre-eclampsia can considered as a two stage disorder (38). This two-stage model suggests that a poorly perfused placenta (stage 1) in turn produces circulating factors leading to the clinical manifestations of the disease (stage 2). In more recent years, further knowledge has been obtained in relation to the pathophysiology of pre-eclampsia and the consensus is that pre-eclampsia is more complex than the two-stage model previously described (39). This led to the idea that maternal constitutional factors (obesity, diet and diabetes), behavioural, environmental and genetic factors, modified by the physiological changes in pregnancy were essential to interact, leading to maternal abnormalities in pre-eclampsia (Figure 1.3) (40).

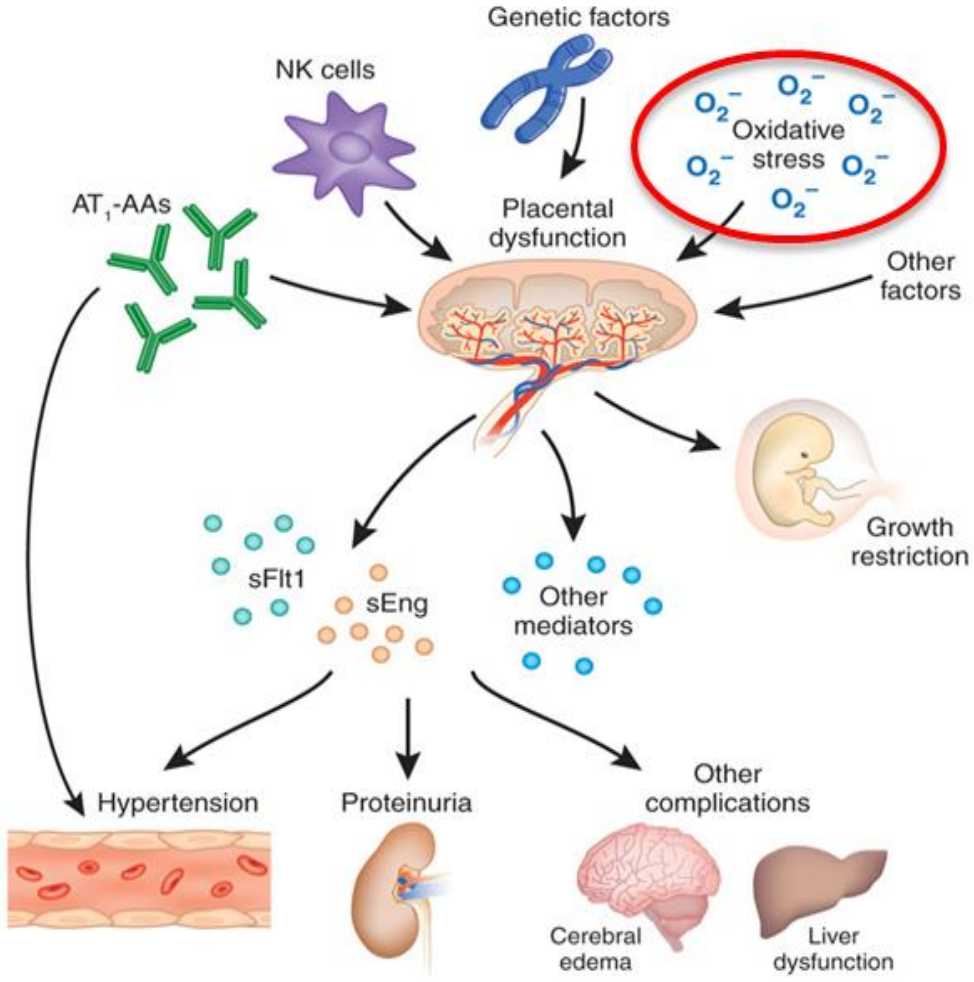

Figure 1.3: Pathogenic mediators that affect placental dysfunction in pre-eclampsia. This figure was generated from Karumanchi et al (41).

1.2.3 Endothelial dysfunction and pre-eclampsia

The key physiologic function of endothelial cells are to control vascular function by detecting circulating blood composition and furthermore providing a physical barrier to the inadequate movement of proteins, ions, water and cells from the blood into the vascular wall (42). Endothelial dysfunction in pregnancy is defined as a lack of enhanced vasodilation of the uterine arteries in early pregnancy. Endothelial dysfunction contributes to all major symptoms of pre-eclampsia. Impairment in remodelling of spiral arteries and poor placentation due to shallow trophoblastic invasion results in deficient placental perfusion (43). The reduced placental perfusion mediated changes release circulating pathogenic mediators such as sFlt-1, sEng, VEGF and ROS, which challenge endothelial cells through different mechanisms, resulting in endothelial dysfunction.

Both vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) and their receptors are important mediators of angiogenesis and placental maintenance. VEGF is widely expressed at the feto-maternal interface and has been established as a major angiogenic factor that mediates endothelial proliferation and vascular permeability by inducing the expression of integrins on endothelial cell (29). Likewise, PIGF mediates pro-angiogenic effects on the feto-placental circulation and supports trophoblast growth. Low levels of circulating PlGF leads the manifestation of clinical disease in pre-eclamptic pregnancies (44). Soluble fms-like tyrosine kinase 1 (sFlt-1) is a splice variant of the VEGF receptor, Flt-1, that lacks

both the transmembrane and cytoplasmic domains of the cell membrane bound receptor. sFlt-1 acts as a soluble receptor that binds PlGF and also binds and deactivates circulating VEGF. The primary receptor for VEGF is VEGF receptor-2 (VEGFR-2) which is essential for normal endothelial proliferation and vascular formation. VEGFR-2 is expressed in endothelial cells, megakaryocytes and haematopoietic stem cells and also in the trophoblast layer in human placenta (41). Research has shown that sFlt-1 directly interacts with VEGFR-2 *in-vivo*, and is significantly more evident in placental tissue from pre-eclamptic patients (45).

Increase in circulating sFlt-1 results in a decrease in the plasma levels of free VEGF and PlGF and these changes are linked with both the occurrence and severity of pre-eclampsia (46-48). Endoglin (Eng) is a type I membrane glycoprotein located on cell surfaces and is part of the transforming growth factor beta (TGF-β) receptor complex and plays a vital role in angiogenesis. Eng is expressed by trophoblast and endothelial cells (49), soluble endoglin (sEng) is a condensed form of Eng. Similar to sFlt-1, sEng is also detected at increased concentrations in placental tissue from pre-eclamptic pregnancies (50). sEng cause endothelial dysfunction by antagonizing TGF-β1 signalling in pre-eclampsia. Proteinuria is one of the primary clinical manifestations of pre-eclampsia. Podocytes are a major source of VEGF production in the glomerulus. To date, there is evidence that VEGF is important in maintaining glomerular endothelial cell health (51), however, with its absence, proteinuria is induced (52, 53). Renal pathologic abnormalities in the form of endotheliosis have been recognised and proteinuria has been commonly viewed as a consequence of endothelial cell swelling. However, renal involvement has been often understood to be in the context of endothelial dysfunction (54).

1.3 Oxidative stress and pre-eclampsia

There is overwhelming evidence that oxidative stress plays a role in the pathophysiology of pre-eclampsia (55-58). Oxidative stress is an imbalance between the production of reactive oxygen species (ROS) and antioxidant defences (59), resulting in increased levels of ROS with resultant damage of cellular components including DNA, proteins and lipids. Uncomplicated healthy pregnancy is characterised by a low grade oxidative stress; there are increased circulating levels of oxidised low-density lipoproteins and a reduction in total antioxidant capacity in pregnant women when compared with non-pregnant women (60). Excessive oxidative stress is generally thought to be involved in the pathology of many pregnancy-related disorders such as fetal growth restriction (FGR), pre-eclampsia and miscarriage (61). Dysfunctional placentation is proposed to provoke an ischaemic environment due to fluctuations in the delivery of oxygen to the fetus, resulting in elevated oxidative stress seen in pre-eclampsia.

By 10–12 weeks' gestation in normal pregnancy maternal blood flow in the placenta causes a local increase in oxygen and elevation in the activity of the antioxidant enzymes (62). However, in pregnancies complicated by pre-eclampsia there is a decrease in antioxidant enzyme activity at the same time in gestation (63). Increased lipid peroxidation as a result of exaggerated oxidative stress plays a vital role in the pathology of pre-eclampsia by provoking endothelial dysfunction. Plasma malondialdehyde (MDA) levels have been found to be significantly higher in pre-eclampsia in comparison with uncomplicated pregnancy (60). Additionally, superoxide dismutase (SOD) antioxidant levels were significantly lower in pre-eclampsia in this study (60).

In recent years, increased levels of cell-free fetal haemoglobin (HbF) has been implicated as a new pathological factor of pre-eclampsia (64). Haemoglobin reacts spontaneously with oxygen generating free oxygen radicals. Haemoglobin and its degradation products are toxic and can lead to oxidative stress in the maternal circulation (65). Recent work has demonstrated elevated levels of several haemoglobin related metabolites including heme, bilirubin and biliverdin in pre-eclampsia patients (66).

1.3.1 Reactive Oxygen species

Reactive oxygen species (ROS) are produced as a by-product of the electron transport chain. ROS are typically recognised as two groups; free radicals and non-radical products. Free radicals include superoxide (O^{2-}), the hydroxyl radical (HO), lipid peroxy-radicals (LOO) and alkoxy-radicals (LO). These are unstable and have a short half-life. Non-radical derivatives are more stable with a longer half-life, however, they can have strong oxidant properties. Non-radicals include hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$) and hypochlorous acid ($HOCl^-$) (59). Superoxide acts as an oxidant as well as a reductant in many biological systems (67) and serves as an origin for many other biologically relevant ROS including H_2O_2, HO radical and $ONOO^-$ (59). H_2O_2 is created by dis-mutation of O^{2-}, this molecule can occur spontaneously, or it can be promoted by superoxide dismutase (SOD).

Hydroxyl radical (HO) is created when O_2 donates one electron to H_2O_2 in a reaction known as the Haber Weiss Reaction. The HO molecule is a highly reactive oxidant that attacks a variety of biomolecules such as lipids, proteins and DNA. Peroxynitrite ($OONO^-$) results from the spontaneous reaction between O_2 and nitric oxide (NO) and is a very strong oxidant and reacts with lipids, DNA and proteins (59). This

molecule reacts and modifies proteins and other cellular structures inflicting oxidative damage on these molecules. OONO- can oxidize lipids, oxidize methionine and tyrosine residues in proteins and oxidizes DNA to form nitroguanine (68). Furthermore, nitroguanine residues are considered as a marker of peroxynitrite induced cellular damage (69).

Redox signalling occurs when a biological system alters in response to a change in the level of ROS or due to the shift in redox state of a responsive group such as dithiol-disulphide (70). The mitochondria are deemed to play a role in redox signalling due to the flux in $O_{2}^{\cdot -}$ generated by the electron transport chain and other metabolic processes in the mitochondria (70). The main ROS involved in redox signalling both to and from the mitochondria is H_2O_2.

1.3.2 Producers of Reactive oxygen species

There are several different sources of ROS including Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase and mitochondria. NADPH oxidases are activated by a variety of physiological and pathophysiological stimuli including inflammatory cytokines, mechanical forces and growth factors. Xanthine oxidoreductase exists in two forms; xanthine oxidase and xanthine dehydrogenase. Xanthine dehydrogenase transfers electrons from hypoxanthine and xanthine to NAD^+ generating NADH and uric acid (59). When a critical cysteine residue is oxidised by peroxynitrite, xanthine dehydrogenase (XDH) is converted to xanthine oxidase. Xanthine oxidase then transfers electrons to oxygen from NADH and uric acid generating O_2^- and H_2O_2. Xanthine oxidase has been shown to contribute to experimental hypertension in animal models, however currently there is limited evidence supporting the role of xanthine oxidase in human hypertension (71).

The natural defence against ROS comprises both enzymatic and non-enzymatic systems. Superoxide dismutase (SOD) is a well-known antioxidant enzyme that prevents free radical mediated injury by metabolising superoxide anions that are known to damage human tissues (72). SOD is categorised into three isoforms, cytosolic Cu, Zn-SOD, and mitochondrial Mn-SOD, which catalyse the dismutation of O^{2-} into H_2O_2 and O_2. In addition to requirements for metal ion cofactors, SOD enzymes also have distinct subcellular localizations. Eukaryotes only express Cu/Zn SODs (in the cytoplasm and extracellularly) and Mn SODs (in the mitochondria).

A decrease in the endogenous SOD antioxidant defence systems weakens the normal metabolic removal of O_2. Additionally, endogenous protein alpha-1-microglobulin acts as a radical scavenger and an antioxidant. It provides protection to tissue and protects the mitochondria from oxidative damage.

Non-enzymatic antioxidants also act as ROS scavengers and include vitamins A, C and E; glutathione, bilirubin and uric acid. Vitamin E is a fat-soluble vitamin and its main role is to protect against lipid peroxidation. Vitamin A (retinol) is crucial for a diversity of physiological processes, including embryonic development and immune system function. Vitamin C scavenges free radicals in aqueous solution. Glutathione is largely known to reduce the lipid peroxidation of cellular membranes (73). Bilirubin has both antioxidant and anti-inflammatory activities, and is inversely correlated with disease risk of the cardiovascular system, such as hypertension (74). Uric acid is a plasma antioxidant capable of scavenging superoxide, hydroxyl radical and singlet oxygen (75) as well as decreasing nitrosylation of tyrosine residues on proteins and is capable of maintaining superoxide dismutase activity (76).

1.3.3 Mitochondria

Mitochondria are rod-shaped organelles that are considered to be the power house of the cell due to their central role in Adenosine triphosphate (ATP) production. The location of the mitochondria in the cell and their size vary significantly depending on the cell type. Mitochondria are actively involved in a number of cellular activities such as proliferation, redox signalling oxidative stress and inflammation. The mitochondrion consists of two phospholipid bilayers which further segregate into four sectors; outer membrane, the inter-membrane space, inner membrane and the matrix (Figure 1.4) (77). The outer membrane contains porins that allow molecules that are less than 5KDa to diffuse freely. The outer membrane acts as a permeability barrier to the cytosolic components. The inner membrane of the mitochondria is the most extensively studied part of the organelle due to its importance in oxidative phosphorylation. The inner membrane is also different from other membranes due to the presence of cristae which allows the surface area to increase and allows for compartmentalisation.

Mitochondria also contain their own genetic material known as mtDNA. Each mtDNA molecule consists of approximately 15000-17000 bp. The mtDNA strands differ in the nucleotide content with the heavy strand being rich in guanine and the light strand being rich in cytosine. mtDNA encodes for a total of 37 genes programmed by the mitochondrial genome (78). The mitochondrial matrix of the cell contains both mitochondrial DNA (mtDNA) and the majority of the enzymes that are required for the production of ATP by oxidative phosphorylation.

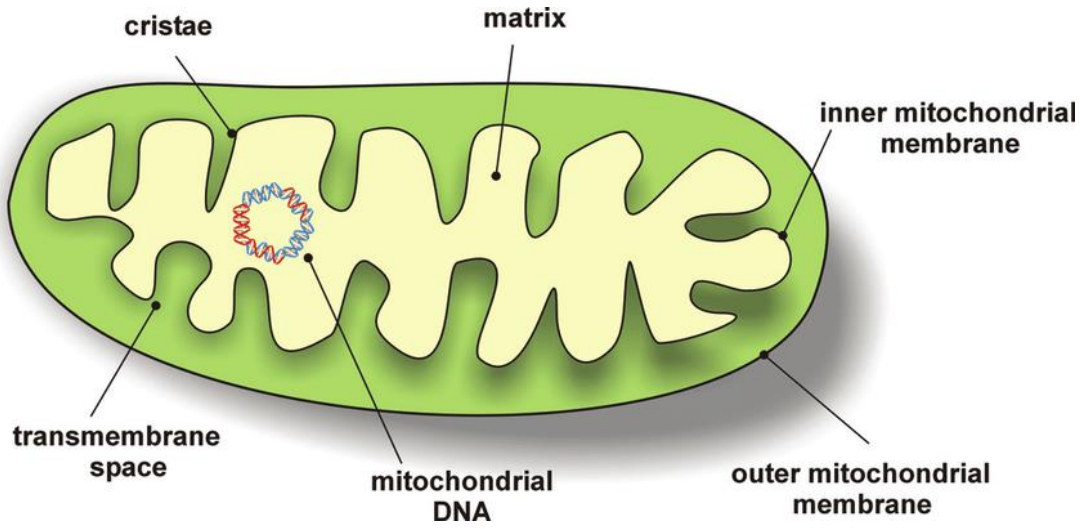

Figure 1.4: Mitochondrial structure and components**.** This figure was generated from Biorender.com

1.3.4 Oxidative Phosphorylation

The main function of mitochondria is the synthesis of ATP by oxidative phosphorylation through the transfer of electrons through multi-subunit complexes (79). Four complex units configure the electron transport chain (ETC) on the inner mitochondrial membrane; complex I, II, III and IV (Figure 1.5). Oxidative phosphorylation starts with the arrival of nicotinamide adenine dinucleotide (NADH) and Flavin adenine dinucleotide ($FADH_2$) from the citric acid cycle. NADH transfers high energy molecules to complex I, while $FADH_2$ transfers high energy molecules to complex II. This transfer of high energy molecules results in loss of electrons from both NADH and $FADH_2$ known as oxidation. The free electrons are transferred to protein complex III and are picked up by cytochrome C which carries the electrons to the final protein complex IV.

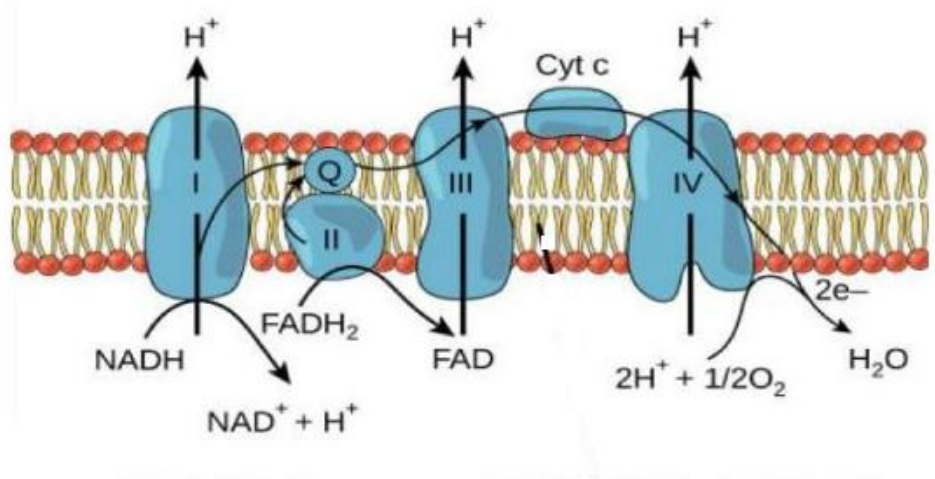

Figure 1.5: Oxidative phosphorylation. This Figure was generated from Biorendar.com

In complex IV, there is a high concentration of protons outside of the inner mitochondrial membrane, resulting in protons being pushed through ATP synthase. This movement of protons causes ATP synthase to spin to produce ATP (79, 80). The ETC generates a proton motive force (pmf) by pumping protons from the mitochondrial matrix to the intermembrane space. When the pmf is high, the ETC slows due to the pump of protons against a stronger electrochemical force, therefore the energy of the substrate oxidation is insufficient to move protons up the gradient. Higher pmf is linked with greater ROS formation, whereas a decrease in pmf can reduce ROS production (81-83).

1.3.5 Mediators of mitochondrial function

Mitochondria are constantly undergoing fission and fusion events, which control their morphology, function and integrity (77, 84). Mitochondrial fission and fusion processes are both mediated by large guanosine triphosphatases (GTPases) (85). Their combined actions divide and fuse the two lipid bilayers that enclose the mitochondria. Fusion is the mixing of the mitochondrial genomes, diluting and in turn protecting against damaged DNA (86). Fission is required for normal mitochondrial function and is essential for growing and dividing cells to provide them with adequate numbers of mitochondria. Furthermore damaged mitochondria are segregated by the fission process to preserve the function of the mitochondrial network (87). Changes in metabolism are linked to the rates of mitochondrial fission and fusion (88). Distorted mitochondrial dynamics have been investigated as potential instigators of mitochondrial dysfunction. Mitochondrial homoeostasis is crucial to maintain efficiency of the mitochondria, therefore exaggerated dysregulation of either the fission or fusion mechanisms could lead to mitochondrial

dysfunction with subsequent elevation in mitochondrial ROS (mROS) generation (77).

The health of mitochondria is regulated by their biogenesis and its orchestrator peroxisome proliferator-activated receptor gamma coactivator alpha (PGC-1α). Disrupted PGC1-α signalling can lead to altered mitochondrial biogenesis and further influence dysregulated metabolic outcomes (89). Mitophagy is the selective degradation of mitochondria by autophagy. This process occurs in defective mitochondria following damage by oxidative stress. Ca^{2+} ions are involved in a number of signalling pathways that regulate mitochondrial function (90). Mitochondria accumulate calcium via membrane-potential driven uniporters and release calcium by electroneutral exchange for sodium or protons (91). Excessive mitochondrial Ca^{2+} uptake establishes the fundamental steps in mROS generation in excitotoxicity (92) and calcium induced toxicity (93), mitochondrial Ca^{2+} overload also plays a role in the mechanisms of oxidative stress in ischemic-reperfusion injury and oxidation injury (94, 95). Many studies have documented that pre-eclampsia is linked with changes in intracellular Ca^{2+} homeostasis (96-98). Further research is needed to examine whether alterations in Ca^{2+} regulation is a consequence in pre-eclampsia due to alterations ion-channel activity because of an imbalance between prooxidants and antioxidants.

Cellular events such as decreased ATP production, calcium dysregulation, increased generation of ROS and mtDNA damage are intrinsically linked to mitochondrial dysfunction (99). Mitochondrial dysfunction has a critical role in the pathophysiology of many human pathologies such as atherosclerosis and diabetes, mainly due to the function of dynamic mitochondria in cellular metabolism. Mitochondrial ROS (mROS) are biologically important mediators in numerous

physiological systems including adaptation to hypoxia and regulation of differentiation (100). Mitochondria have significant DNA repair capacity, however mtDNA are vulnerable to oxidative damage due to its proximity to the electron transport chain (ETC) as well as the lack of protection by histones and chromatin (99). Mitochondrial dysfunction and mtDNA damage are often linked to diseases associated with exaggerated oxidative stress including hypertension and atherosclerosis (101). mtDNA is therefore often used as a biomarker of mitochondrial dysfunction. Furthermore, mtDNA lesions have been found in the circulating cells and hearts of patients that had coronary heart disease (102).

1.3.6 Evidence of mitochondrial dysfunction in pre-eclampsia

Mitochondrial activity is fundamental in pregnancy as it maintains the metabolic activity of the placenta (103). Research has shown elevated mitochondrial lipid peroxidation and increased evidence of susceptibility to oxidation in mitochondria of pre-eclamptic placentas (104). Furthermore, increased activity of the placental mitochondrial electron transport chain in preterm pre-eclamptic patients compared to normotensive controls has been reported (105). Changes in the pre-eclamptic placental proteomes may explain the significance of mitochondria in the development of pre-eclampsia. Shi et al carried out a comparative proteomic analysis of mitochondrial proteins in normal placentas compared to severe pre-eclampsia placentas. Here they found 26 differentially expressed mitochondrial proteins between the two groups. Furthermore, they illustrated the majority of these proteins were found to be involved in many biological processes including oxidative stress, ROS generation and mitochondrial damage (106). As well as this, they also observed degenerative changes and swollen mitochondria in the placentas from pre-eclamptic women. Research has shown disruption in oxygen consumption rate in trophoblast

cells isolated from pre-eclamptic placentas compared to normotensive placental trophoblasts (107).

Hypertension is a central clinical characteristic of pre-eclampsia and elegant work by Dikalova et al, showed that mitochondrial dysregulation and resultant elevation in mROS resulted in elevation in hypertension *in vivo* (108). Research carried out by McCarthy et al, showed that mROS is elevated in HUVEC cells treated with plasma from pregnancies complicated with pre-eclampsia. Similarly, they showed that plasma from pre-eclamptic women significantly reduced mitochondrial respiration in treated HUVEC. These results highlight that mROS plays a role in mediating endothelial dysfunction in pre-eclampsia (109).

1.4 Role of Inflammation in pre-eclampsia

During normal pregnancy, three different immunological phases can be characterised. These include; a pro-inflammatory environment during embryo implantation, placentation and early stage of pregnancy, an anti-inflammatory milieu during mid trimester and a pro-inflammatory environment in third trimester and end of pregnancy (110). The innate immune system acts as both a protector and effector during pregnancy. As a protector, the innate system releases cells such as macrophages, dendritic cells, natural killer cells and neutrophils which protect the mother from invading pathogens. Furthermore, gamma delta (γδ) T cells are upregulated during pregnancy in order to protect the mother against pathogens while the adaptive immune system is dampened so as not to prompt a specific immune response toward the fetus. T cells have been examined extensively in healthy and complicated pregnancies. T-helper cells mediate their effects by the secretion of cytokines. Th1 cells produce pro-inflammatory cytokines including interferon IFN-γ and tumour necrosis factor (TNF)-α, which are involved in cell-mediated responses, whereas Th2 cells produce predominantly anti-inflammatory cytokines such as IL-4, IL-5, IL-10 (111).

Cytokines are proposed to control trophoblast behaviour by either modulating proliferation and migration or by inducing trophoblasts to differentiate into a non-invasive phenotype (112). The exact role of cytokines in the development of pre-eclampsia is yet to be fully elucidated but they may be involved in either the initial placental insult or the maternal response to initial placental insult. For example, TNF-α has a role in the maternal vascular dysfunction by decreasing the availability of the vasodilator nitric oxide (NO) by reducing eNOS expression in the endothelial cells thereby reducing NO formation (113).

Cytokine levels in pre-eclampsia have been a constant source of conflict in many publications in regard to its association with the disease. Several studies have shown an increase in pro-inflammatory cytokines in serum and plasma of women with pre-eclampsia such as TNF-α, IL-6, IFN-γ and IL-1β and anti-inflammatory cytokines, IL-10 (114-125) (Table 1.1).

TNF-α promotes apoptosis and leakage of the endothelial vessels, leading to systemic endothelial activation and thereby signs associated with pre-eclampsia. There was elevation in pro-inflammatory cytokine TNF-α seen in the majority of cases with the exception of one study carried out by Kumar et al, where they saw a decrease in TNF-α expression in pre-eclampsia (111). In this study they examined TNF-α levels in the second trimester of women who went on to develop pre-eclampsia (n=14) compared with normotensive pregnancies (n=162) as well as examining TNF-α levels 24 hours after delivery. These lower levels of TNF-α seen in pre-eclampsia compared with controls may be due to measuring in the second trimester and before the manifestation of the disease (111). Furthermore in this study, IL-10 showed lower levels in pre-eclampsia compared to normal pregnancy (111), however, IL-10 was increased in two further studies (120, 126).

IL-10 affects the interaction between trophoblasts and endothelial cells during placentation of the placenta and undesirably regulates the levels of TNF-α and IL-6. Szarka et al, examined IL-10 levels in the third trimester in 60 women who developed pre-eclampsia and found a significant increase in levels compared to controls (126). Similarly, Silvia et al, found a significant increase in IL-10 levels in women in the third trimester who had pre-eclampsia (n= 40) compared with controls (n=36) (120).

IL-6 is a multi-functional pro-inflammatory cytokine that is produced by the activated vascular endothelial cell and placenta (127) and it functions to control the immune

response and inflammation. IL-6 was seen to be increased in all studies examining levels in the third trimester of women with pre-eclampsia compared to healthy controls (118, 120, 121).

Table 1.1: Inflammatory cytokines in pre-eclampsia

Study	Serum	TNF-α	IL-6	IL-1β	IL-10	INF-γ
Kumar et al. 2013	x	↓			↓	↓
Roudsari et al. 2009	x	—				
Founds et al. 2008	x	↑				
Szarka et al. 2010	x	↑	↑		↑	
Koçyıgıt et a. 2004	x	↑	↑	↑		
Silva et al. 2013		↑	↑		↑	
Xiao et al. 2012	x		↑			
Hamai et al. 1997		↑				
Williams et al. 1999		↑				
Sibai et al. 2009	x	↑				

1.4.1 Neutrophils and pre-eclampsia

Neutrophils have been viewed as short-lived effector cells of the innate immune system, where their primary role is in the clearance of extracellular pathogens. Neutrophils spontaneously or following appropriate stimulation, have been shown to produce many cytokines, chemokines and angiogenic factors. Neutrophils are also known mediators of the T helper 17 pathway of resistance to pathogens. Uncomplicated healthy pregnancy is characterised by the presence of neutrophils at the feto-maternal interface from the first trimester (124).

In pre-eclampsia, neutrophilia is further increased compared to uncomplicated pregnancy (128), and there is extensive evidence that neutrophils are activated in both the peripheral blood and decidua of pre-eclamptic patients (129, 130). Neutrophils have been strongly linked with vascular dysfunction in women with pre-eclampsia, due to their ability to form neutrophil extracellular traps (NETs) and adhere to the endothelium in high concentrations (131). Neutrophil extracellular trap formation is triggered when mtDNA binds to receptors, including Toll-like receptor 9 (TLR9) on the surface of the neutrophils. Increased NETs formation is seen in pre-eclampsia and is proposed to significantly contribute to the underlying pathology of pre-eclampsia (132).

1.4.2 TLR9 activation of the neutrophil mediated innate immune response

Toll-like receptors (TLRs) are fundamental components of the innate immune system that recognise both microbial ligands and host products circulating during tissue damage. TLRs specifically bind to highly conserved protein sequences, also known as pathogen associated molecular patterns (PAMPs) (133). However, some TLRs bind to endogenous non-pathogen associated ligands, also known as damage-associated molecular patterns (DAMPs). The activation of TLRs via PAMPs and/or DAMPs results in a pro-inflammatory innate immune response.

Endogenous DAMPs are derived from many sources such as the endoplasmic reticulum, nucleus, plasma membrane, cytosol and mitochondria (134). Mitochondrial DAMPs (mtDAMPs) express two unique molecular signatures that are evolutionarily conserved from their bacterial origin, N-formyl peptides and mitochondrial DNA (mtDNA). Mitochondria evolved from saprophytic bacteria to become intracellular organelles, hence mtDNA is structurally similar to bacterial

DNA and share unmethylated CpG DNA repeats (135). Bacterial DNA is a known ligand of TLR9. Subsequently, the molecular motifs expressed by mtDNA also activate the TLR9 signalling pathway and induce an innate immune response (136). In normal pregnancy and pre-eclampsia, TLR9 is expressed in the vascular endothelium and trophoblasts. There is evidence of increased TLR9 expression in both placental tissue (137-140), and vascular endothelial cells in pre-eclampsia (141), implying that TLR9 signalling may be implicated in the development of placental dysfunction and the pathogenesis of pre-eclampsia.

1.4.3 Neutrophil Activation markers

1.4.3.1 Calprotectin

Calprotectin is a calcium and zinc-binding protein found in cells of myeloid origin, such as neutrophils and monocytes and plays a role in many physiologic functions including the neutrophil defence against bacterial infections. Calprotectin is also known as S100A8/S100A9 (142). This protein has been shown to be increased in patient plasma during bacterial infections (143). Calprotectin production is increased in cytotrophoblasts, placental-tissue macrophages, endothelial cells during the first and second trimester of pregnancy (144). Furthermore, there is a decrease in calprotectin expression in the placenta at the end of pregnancy. In pre-eclampsia, calprotectin concentrations are found to be 2-fold higher in maternal plasma when compared to controls (144).

1.4.3.2 Myeloperoxidase

Myeloperoxidase (MPO) is a lysosomal enzyme which is produced and released by activated neutrophils. MPO catalyses the formation of reactive oxygen intermediates such as hypochlorous, hypobromous and hypothioxyanous acids (145, 146). MPO has been found to be implicated in the pathogenesis of numerous inflammatory diseases such as kidney disease, rheumatoid arthritis and metabolic syndromes. Furthermore, this enzyme has been linked to vascular injury and is associated with the pathogenesis of cardiovascular disease, largely due to its oxidative and pro-inflammatory properties (147, 148). MPO is associated with nitric oxide and its impairment of bioavailability (148, 149) causing nitration of protein tyrosines (150, 151). Nitric oxide is known to be reduced in pre-eclampsia, this in turn gives rise to elevated blood pressure due to the imbalance between vasodilators and vasoconstrictor influences on the smooth muscle suggesting that MPO contributes to vascular dysfunction during acute inflammation by modulating endothelial NO bioavailability (152).

MPO has been shown to accumulate on the surface of neutrophils in pregnant women, therefore enhancing the metabolic activity and oxidant production of neutrophils throughout pregnancy (153). Furthermore, MPO levels have been found to be increased in maternal circulation and placental tissue of women with pre-eclampsia (154-156). However, other studies have failed to show this increased MPO levels in women with pre-eclampsia (157, 158). This may be due to sampling period during pregnancy, those with no evidence of increased MPO were sampled at 20 weeks before the manifestation of pre-eclampsia, while those reporting increased MPO were taken in the third trimester.

1.4.3.3 Matrix Metalloproteinase-8

Matrix Metalloproteinase (MMPs) consist of 23 zinc and calcium dependent proteases that cause degradation of different components of the extracellular matrix. The levels of MMPs are generally very low in normal adult tissues, however, their expression is increased in tissues with inflammation or undergoing active remodelling (159, 160). Increased expression and activity of MMPs are linked to pathological conditions such as chronic inflammation and cancer (161-163). MMPs participate in tissue turnover at the beginning of the pregnancy and are important for the cytotrophoblast invasion of uterine spiral arteries. MMPs also play a crucial role in the activation of labour at term (164, 165). In recent years, MMPs have been an emerging area of interest in relation to the role they play in pre-eclampsia. MMP-8 is known as neutrophil collagenase or collagesase-2. Neutrophil collagenase was initially described in the 1990s when it was cloned from neutrophils taken from a patient with granulocytic leukaemia (166). MMP-8 expression is inducible and upregulated by many inflammatory cytokines including TNF-α and IL-1β (167). Recent research shows strong evidence that MMP-8 plays an important role in mediating endothelial cell angiogenesis (168). Furthermore, endothelial dysfunction is a pathogenic characteristic of pre-eclampsia, dysregulated MMP8 expression may play a crucial role in the disruption of angiogenesis in pre-eclampsia leading to endothelial dysfunction.

1.4.3.4 IL-8

Chemokines, a subset of cytokines, specific in their ability to attract and activate immune cells, may be particularly important mediators of pregnancy. IL-8 is a known pro-inflammatory chemokine and increased levels have been associated with pregnancy complications including IUGR (169) and preterm delivery (170). IL-8 is also a neutrophil chemoattractant and activator, is produced by many tissues such as the placenta (171, 172). The role of IL-8 is to promote movement of neutrophils across endothelial monolayers and towards the site of inflammation. Furthermore, IL-8 regulates endothelial cell proliferation, angiogenesis, and tumour growth as well as participating in placental infection (173-175). In neutrophils, IL-8 triggers the secretion of superoxide anions and lysosomal enzymes which indirectly expand the permeability of blood vessels (176).

Similarly, when compared with healthy uncomplicated pregnancies, the majority of studies report increased maternal serum and plasma levels of IL-8 in pregnancies complicated by pre-eclampsia (177, 178). A recent study reported a significant increase in IL-8 in early gestation (5-15 weeks') in women who subsequently went on to develop pre-eclampsia. However, a limitation of their study was the small sample size (n=9) (179). Other studies investigating IL-8 throughout pregnancy reported no significant increase evident in the second trimester (180, 181) but a significant increase in IL-8 was evident in the third trimester in women with pre-eclampsia compared to healthy controls (182, 183) therefore supporting the role of IL-8 in mediating an inflammatory response in pre-eclampsia.

1.5 Therapeutic Potential of Antioxidants in Pre-eclampsia

1.5.1 Vitamins

Vitamin C and Vitamin E are exogenous antioxidants that down-regulate NADPH oxidase, a major source of ROS in the vasculature, while upregulating eNOS, leading to vasorelaxation and a reduction in blood pressure (56). The therapeutic potential of these particular antioxidants in the prevention of conditions associated with oxidative stress is supported by an extensive evidence base comprising of experimental studies (184), observational studies (185) and small clinical studies (186). However, in stark contrast, large scale appropriately powered randomised clinical trials (RCTs) of antioxidants as a treatment for pre-eclampsia have been disappointing. The VIP trial (Vitamin C and vitamin E in pregnant women at risk for pre-eclampsia) was a RCT of vitamin C and vitamin E in 2410 women identified as being at an increased risk of pre-eclampsia (187). This study assigned women with 1000mg vitamin C and 400 IU vitamin E or matched placebo daily from the period of the second trimester of pregnancy until delivery. This trial concluded that supplementation with vitamin C and vitamin E did not prevent pre-eclampsia. However, a secondary outcome from this clinical trial showed that vitamin supplementation increased the rate of babies born with low birthweight (187).

Another multicentre, randomised, double-blinded trial was carried out on 10,154 nulliparous women looking at daily supplementation of 1000 mg of vitamin C and 400 IU of vitamin E to prevent complications in pregnancy (188). The primary outcome for this clinical trial was to reduce severe pregnancy-associated hypertension or severe or mild hypertension with many clinical outcomes such as eclamptic seizures. Supplementation with vitamin C and E did not reduce the incidence of gestational

hypertension or pre-eclampsia in low risk nulliparous women (188). In comparison with the VIP trial, this study did not find significant differences between groups in the rates of low birth rate and stillbirth.

The administration of Vitamins C and E for prevention of pre-eclampsia in women with type 1 diabetes (DAPIT) was a randomised placebo-controlled clinical trial in the UK and Northern Ireland running from 2003-2008 assessing vitamin supplementation for the prevention of pre-eclampsia in women with type 1 diabetes (189). Women in this study were randomly allocated to receive 1000 mg of vitamin C and 400 IU vitamin E or matched placebo every-day from between 8 weeks' gestation and 22 weeks' gestation until delivery. This study showed no significant differences between vitamin and the placebo groups for any of the maternal outcomes. However, plasma ascorbate concentrations for both vitamin-treated groups were significantly higher in comparison to the placebo group at 26 weeks' gestation and 34 weeks' gestation with a low antioxidant status at baseline (189). In summary, in contrast to a significant supportive pre-clinical research base, these large clinical trials suggest that vitamin antioxidant therapy is ineffective in the treatment of disorders such as pre-eclampsia. An explanation into why these antioxidants have been unsuccessful may be due to them being sequestered in the cytosol and not specifically reaching the mitochondria, therefore failing to reduce the oxidative damage.

1.5.1.2 Mitochondrial-Targeted antioxidants

Currently, mitochondria are being recognised as key mediators in many unexpected areas of biomedical science. Mitochondria are the primary source of ROS, therefore, by directly targeting the mitochondria, it provides an opportunity to treat conditions precipitated by exaggerated oxidative stress. To date, two pharmacological strategies have been proven to be useful in targeting small molecule antioxidants to mitochondria; firstly, conjugation to lipophilic cations (190) or secondly incorporation into mitochondrial-targeted peptides (191). Lipophilic cations such as triphenylphosphonium (TPP) derivatives are rapidly and extensively taken up by mitochondria (192). This is due to the mitochondria being negatively charged, therefore using a positively charged lipophilic cations permits direct targeting of the mitochondrial. To date, mitochondrial targeted antioxidants have been examined for the use of many mitochondrial diseases. In particular, research carried out by Stealth Therapeutics using elamipretide, a peptide compound that readily penetrates cell membranes, and targets the inner mitochondrial membrane where it binds reversibly to cardiolipin (193). This peptide increases mitochondrial respiration, improves electron transport chain function, ATP production and reduces formation of pathogenic ROS levels (193-195).

The most studied mitochondrial-targeted antioxidant has been the TTP-modified ubiquinone MitoQ, which has shown efficacy in a number of animal models and also in human studies (196). This compound is largely absorbed by the mitochondrial matrix facing surface of the inner membrane, therefore protecting the components involved in oxidative phosphorylation (197). The first study to look at the protective effects of MitoQ in a rat model of cardiac ischemia/reperfusion (I/R) injury (198). In this study MitoQ was protective against heart dysfunction, tissue damage as well as

mitochondrial function. Another study examined the effects of MitoQ on blood pressure in spontaneously hypertensive rats and showed a reduction in blood pressure (199). The research carried out in animal studied highlighted MitoQ as an attractive therapeutic intervention in human diseases. Phase 1 trials of MitoQ showed good pharmacokinetic behaviour with oral dosing at 80mg (1mg/Kg) (196). MitoQ was administered to patients with Parkinson's disease in the PROTECT study, a double blinded placebo-controlled study ran over 12 months. 128 newly diagnosed untreated patients with Parkinson's disease were enrolled into the study. This study showed that treatment with MitoQ did not slow down the progress of Parkinson's disease (200).

Similarly, MitoQ was used for the treatment of chronic Hepatitis C in the CLEAR study (201). This study was a phase II study carried out due to evidence of the pathological role of increasing oxidative stress and subsequent mitochondrial damage in liver damage. The participants received either 40 mg or 80 mg or placebo for 28 days. This study reported that MitoQ significantly decreased plasma alanine transaminase and aspartate aminotransferase in patients with chronic HCV infection suggesting that MitoQ can reduce liver damage in hepatitis C viral infection. Therefore, mitochondrial targeted antioxidants have shown to have the potential to decrease mitochondrial oxidative damage in a range of diseases as well as being safe for human consumption.

1.5.1.3 MitoTempo

MitoTempo is a mitochondrial targeted superoxide dismutase antioxidant mimetic. MitoTempo has the ability to pass through lipid bilayers easily and accumulate

selectively in mitochondria (202). MitoTempo combines the antioxidant piperidine nitroxide (Tempo) with the lipophilic cation triphenylphosphonium (TPP) for mitochondria targeting (203). Recent work has established that MitoTempo accumulates in the mitochondria by increasing superoxide dismutation and without affecting cytoplasmic dismutation in endothelial cells (108). This study provided evidence that scavenging of mitochondrial O_2^- improves endothelial function and reduces hypertension. They showed that treatment with MitoTempo in two murine models of hypertension reduced blood pressure and substantially improved endothelium-dependent vasodilation. In comparison, MitoTempo had no effect on blood pressure in normotensive animals.

In recent years, research using the SCOPE (Screening for Pregnancy Endpoints) study, showed that deleterious plasma mediators present in pre-eclampsia, generate increased mitochondrial-specific superoxide production in HUVEC cells (109). Additionally, this increase in mROS production was shown to provoke vascular dysfunction and inflammation. The authors expanded this study and reported that pre-treatment with MitoTempo prevented the observed increase in mROS production following plasma incubation.

Recently a study by Vaka et al examined MitoTempo as a treatment for pre-eclampsia using the RUPP model (204). This study established that treatment with MitoTempo attenuated elevated blood pressure in the RUPP rats, in addition to improving fetal outcomes. Furthermore, placental complex activities, expressions and respiration rates were significantly reduced and mROS was increased in RUPP compared to normal pregnant mitochondria.

1.5.1.4 L-Ergothioneine

Ergothioneine (ERG) is a dietary water-soluble amino acid which is derived from histidine and was discovered in 1909 by Charles Tanret (Figure 1.6). In humans, ERG has been shown accumulate in many cells and tissues in high concentrations, such as erythrocytes, liver, kidney, bone marrow and seminal fluid (205-207). There have been many physiological roles of ERG proposed such as a direct antioxidant (208-212) cation chelation (210, 213), as well as regulation of gene expression and immune regulation.

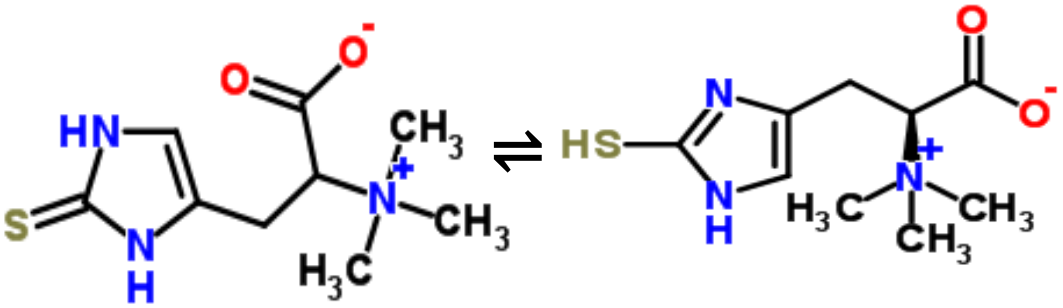

Figure 1.6: Chemical structure of Ergothioneine. It exists predominately in the thione (left hand side) form as opposed to the thiol (right hand side) form, ensuring its resistance to autoxidation.

ERG crosses the intestinal epithelium through the organic cation transporter novel type 1 (OCTN1), which is currently known as solute carrier family 22, number 4 (SLC22A4) (214). Organic cation transporters function primarily in the elimination of drugs in tissues such as kidney, intestine, liver and placenta by mechanisms dependent on or independent of sodium-gradient, pH or membrane potential (215). Previously, SLC22A4 had been considered to be a carnitine transporter, however research from Grundemann et al, established that the observed rates were very low

(216). Using untargeted metabolomics, this group found when comparing HEK 293 cells with or without extra clones SLC224A, ERG was transported 100 times quicker than carnitine. The localisation of ERG is somewhat less clear, however, many papers have focused on its mitochondrial accumulation and localisation (217, 218).

There is strong evidence to support an antioxidant role for ERG, with numerous *in vitro* studies highlighting its ROS scavenging capacity (210, 211, 219-222). To date, research has shown that a reduction in ERG concentration in HeLa cells' resulted in a significant increase in the oxidative burden on mitochondrial mechanism's (223). However, even though the antioxidant role of ERG has been well established *in vitro*, this is a paucity of in vivo studies. There is some evidence *in vivo* to suggest that ERG acts as an antioxidant in animal models of lung injury (224) and ischaemia/reperfusion injury (225). To date, the role of ERG has been examined in chronic inflammatory diseases (226, 227) highlighting the association between SLC22A4 and Crohn's disease, neurodegeneration (228) where ERG has been shown to be decreased in patients with Parkinson disease and cardiovascular disease (229, 230) by protecting against endothelial dysfunction in human brain microvascular endothelial cells (HBMEC's). To date, studies have investigated the reproductive safety in Sprague Dawley rats, and have found no adverse effects (231). Similarly, supplementation of ERG to diabetic pregnant rats for the first 11.5 days of gestation, decreased the embryo malformations in comparison to non-diabetic controls (229).

Currently, only one study has investigated the pharmacokinetics of ERG administration in humans (232). This study administered 5mg or 25mg dose of ERG to healthy human subjects every morning for one week. Plasma ERG was ardently absorbed and retained by the body with significant elevations in plasma and whole

blood concentrations, and relatively low urinary excretion. There were no reported adverse effects from the administration of ERG in humans. Following ERG administration, decreasing trends in biomarkers of oxidative damage and inflammation were evident. In addition, no changes were observed in liver and kidney function tests and lipid profiles from administration of ERG demonstrating its safety (232). ERG has received GRAS (generally regarded as safe) status, received EC authorisation and was launched to market as a dietary supplement in 2016.

There are a number of studies which administered high-doses of ERG and have indicated that ERG is safe for mammalian consumption at levels far in excess of those likely to be encountered in foodstuffs (232-235). It also lacks any detectable mutagenicity or genotoxicity in such assays, even at very high doses. ERG has been declared safe by relevant Committees such as the European Food Standards Agency (236, 237).

The evidence from current studies investigating the uptake and retention of ERG imply that this amino acid has an important physiological role but potentially only under the conditions of oxidative stress. The potential of ERG to accumulate in high oxidative stress organs makes this a promising target for a viable therapy for pre-eclampsia (211). In pre-eclampsia, data suggests that erythrocyte ERG levels are increased (238). This could be due to increased SLC224A expression in response to inflammatory cytokines seen in pre-eclampsia (209). Despite the rarity of a defined role for ERG in mediation of mitochondrial metabolism, the strong antioxidant effects and its localisation to mitochondria suggest that ERG should have a considerable effect of mitochondrial function.

1.6 Animal models of pre-eclampsia

Our understanding of the pathophysiology of pre-eclampsia has greatly improved in the last two decades. Despite this, development of interventions to prevent pre-eclampsia are severely lacking. Therefore, to improve both our understanding of the disorder and the development of new therapeutic strategies, well-validated animal models of pre-eclampsia are required. The benefit of performing these *in vivo* studies is that they allow an insight into complex interactions that underlie pre-eclampsia that *in vitro* experimentation, while valuable, are limited due to the heterogeneous nature of the condition. Currently, there are no *in vitro* systems available, which mimic human pre-eclampsia. Indeed even isolation of cells involved in pre-eclampsia doesn't reflect the pathogenesis of the disease, as central to the progression of the disease is cell-cell interactions and cell signalling (239). Animal models of pre-eclampsia have facilitated the examination of many of the pathophysiological mechanisms that underlie the development of pre-eclampsia.

Pre-eclampsia is thought to originate spontaneously in women, subsequently animal models of pre-eclampsia have obvious limitations. Many animal models have been recommended based on the apparent causes and mediators of pre-eclampsia. These models include the placental ischaemia model (reduced uterine perfusion pressure model) (240, 241), nitric oxide abnormalities (Nitric oxide synthase knockout mice) (242), impaired angiogenesis (sFlt-1 infusion models) (243) and renin angiotensin abnormalities (transgenic models) (244). An ideal model of pre-eclampsia would display all the phenotypic characterisations seen in women such as hypertension, proteinuria, endothelial dysfunction, and an imbalance of angiogenic factors, therefore this makes *in vivo* models very hard to replicate. The most closely related

phenotype to that of human pre-eclampsia that can be replicated, is the reduced uterine perfusion pressure (RUPP) rat model.

1.6.1 Reduced uterine perfusion pressure (RUPP) model

The Reduce Uterine Perfusion Pressure (RUPP) model is one of the most published models for studying pre-eclampsia. This model has been examined in many animals including dogs (245, 246), sheep (247, 248), rabbits (241), primates (249, 250) and rats (251-253). The most well recognised and characterised RUPP model is in the rat. The RUPP model of pre-eclampsia was adapted by Crews and colleagues (254), in relation to the gestation time and the clips size and position. On gestational day 14, pregnant rats undergo surgery, which entails the clipping of aorta above the iliac bifurcation (Figure 1.7) One surgical clip is placed on both the left and right ovarian arteries that supply the uterus to prevent compensatory flow.

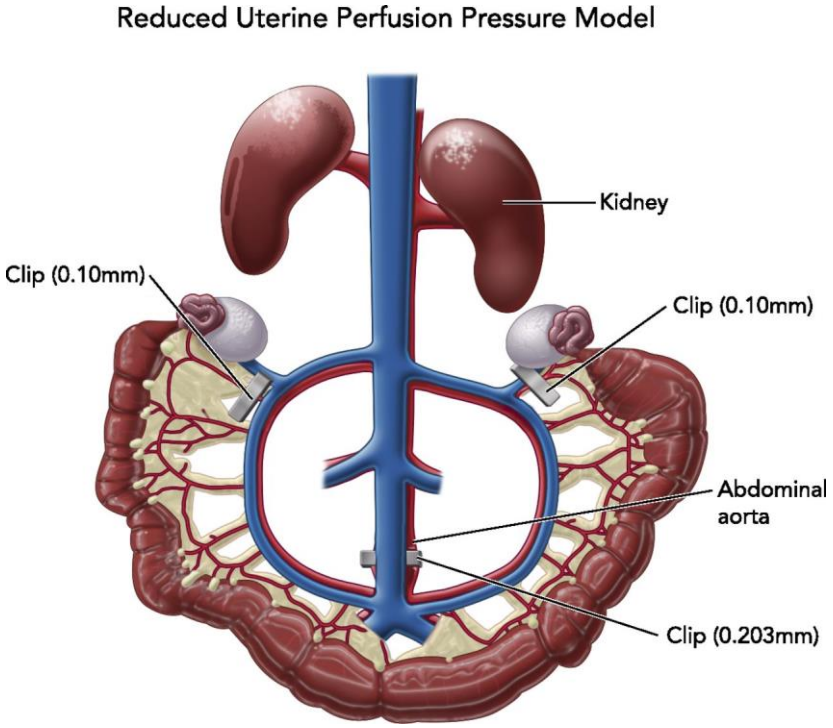

Figure 1.7: Induction of reduced uterine perfusion pressure (RUPP) model in pregnant rats. In the rat RUPP model, on day 14 of gestation, a silver clip with a 0.203-mm internal diameter is placed around the aorta right above the iliac bifurcation, and silver clips with 0.1 mm internal diameter were placed around the left and right uterine arcade at the ovarian artery.

The RUPP rat model has the closest phenotype to that evident in women with pre-eclampsia as it possesses a number of physiological features of pre-eclampsia including hypertension (20-30 mmHg increase in mean arterial blood pressure) and proteinuria (255). Furthermore, IUGR also occurs in the pups in this model illustrating a decrease in litter size and pup weight (253, 256). Inflammatory cytokines TNF-α and IL-6 are increased in the RUPP model, similar to women who develop pre-eclampsia (257). Anti-angiogenic factors including sFlt and sEng are

important in the development of pre-eclampsia in women. Similarly, the RUPP model also displays an increase in plasma and placental sFlt and a decrease in PlGF and VEGF (258). The RUPP model also mimics endothelial dysfunction as evident in pre-eclampsia (254). Evidence has shown impaired vasorelaxation in resistance vessels from RUPP compared with control rats (259). Similarly, plasma from RUPP rats reduced the vasorelaxant response in normal pregnant rats. Therefore, the RUPP-rat model has numerous features of pre-eclampsia evident in women and provides a distinguished model for the investigation of novel therapeutic targets for the treatment of pre-eclampsia.

1.6.2 Challenges of Clinical Trials in pregnancy

The uniquely vulnerable nature of the pregnant and new-born population poses significant logistical, ethical, and regulatory challenges, which have limited the discovery of biomarkers and other predictors for adverse outcomes and the development of diagnostics and therapeutics in general. To date, there are limited number of clinical trials and human studies carried out in regard to pregnancy. Thalidomide was used to treat morning sickness and could be bought over the counter. Pregnant woman who took thalidomide as a treatment for morning sickness had a detrimental effect on the unborn fetus, which resulted in malformation of the limbs. Other defects included malformed eyes and hearts, deformed gastrointestinal and urinary tracts, blindness and deafness.

In more recent years, there was a multisite clinical trial using sildenafil as a potential treatment for IUGR (261). In this instance, extensive research was carried out in many *in vivo* models which had shown promising data for the potential of this drug to treat IUGR (262-265). Sildenafil, a phosphodiesterase type 5 inhibitor, potentiates

the actions of nitric oxide, which leads to vasodilatation of the uterine vessels and might improve fetal growth in utero. However, in this clinical trial, the Dutch site reported the Sildenafil intervention resulted in 11 infant deaths due to lung related problems. Therefore, this study was terminated.

Another clinical trial was carried out recently in regards to the use of statins for the treatment of pre-eclampsia, the StAMP trial. This clinical trial was carried out in women diagnosed with pre-eclampsia according to the ISSHP guidelines with a singleton pregnancy. Women were given 40mg pravastatin or an identical placebo, once per day until their baby was born. (ref)

While pre-clinical data from both animal models and *in vitro* experimentation were deemed successful to proceed humans, unfortunately there were still adverse effects seen in both StAMP or Sildenafil trial. Nonetheless, currently there is no treatment for pre-eclampsia and this thesis work will aim to address the indisputable need for an effective therapy for pre-eclampsia.

1.7 Summary

Pre-eclampsia is a complex multisystem vascular syndrome characterised by the onset of hypertension on or after 20 weeks' gestation. It affects 5% of first-time mothers and globally this disorder causes around 70,000 maternal deaths annually. As described throughout this introduction, pre-eclampsia is thought to result from inadequate invasion of the spiral arteries, resulting in deterioration of the uteroplacental perfusion. This therefore leads to intermittent fluctuations in oxygen delivery and exposes the placenta to oxidative stress (266). In pre-eclampsia, the properties regulating the placental oxidative state are aggravated, causing an increase in production of deleterious inflammatory factors and in turn, vascular dysfunction. The mitochondria are regulators of cell death and are the main cellular source of ROS. As previously described, excessive mROS is liked to mitochondrial dysfunction (136, 267). Research has described a positive association of mtDNA in maternal circulation with the risk of pre-eclampsia (268). Further to this, research has shown that the mitochondria may provide a possible trigger to activating the innate immune system (269). Previous work from our lab has hypothesised that mtDNA may act as DAMP's and instigate an innate immune response by binding to TLR9 (109, 270). As previously mentioned, there is substantial evidence indicating a potential role for dysregulation of mitochondrial function in mediating the clinical characteristics of pre-eclampsia (268, 271, 272). Currently there is no treatment for pre-eclampsia except delivery of the placenta and the baby, with the resultant risk of iatrogenic prematurity and significant neonatal morbidity and mortality. As a result, intensive research endeavours have focused on defining the molecular mechanisms of pre-eclampsia and thereby expediting identification of new therapeutic targets for

this condition. In pregnancies affected by pre-eclampsia there is an exaggerated oxidative stress phenotype with a correspondent deficient antioxidant response. The therapeutic potential of vitamin antioxidants in the prevention of pre-eclampsia was supported by extensive research, however, ultimately, they were unsuccessful in clinical trials. In more recent years, mROS has been identified as playing a prominent role in modulating hypertension (108). This highlights the need for a specific targeted approach to oxidative stress in pre-eclampsia. L-Ergothioneine as previously described, is a water-soluble amino acid is currently FDA approved, increasing its potential and ease of translation into the clinic.

1.8 Hypothesis

Targeting mROS and oxidative stress seen in pre-eclampsia with a specific mitochondrial targeted antioxidant will alleviate pre-eclampsia phenotype.

1.9 Objectives

1. To characterise the role of mitochondrial dysfunction in women with pre-eclampsia compared to uncomplicated pregnancies.

2. To delineate the role of mitochondrial dysfunction in provoking a neutrophil-mediated immune response in pre-eclampsia.

3. To examine the mechanistic role of mitochondrial targeted antioxidant L-ergothioneine in modulating mitochondrial function in an *in vitro* model of placental oxidative stress and its role as a therapeutic target of pre-eclampsia.

4. To analyse the therapeutic potential of mitochondrial targeted antioxidant L-ergothioneine *in vivo* using the RUPP rat model of pre-eclampsia.

Chapter 2: Exploring the role of mitochondrial dysfunction in the pathophysiology of pre-eclampsia.

Williamson RD, Fergus P McCarthy, Ali S. Khashan, Ainhoa Totorika, Louise C Kenny, Cathal McCarthy. Exploring the role of mitochondrial dysfunction in the pathophysiology of pre-eclampsia. Pregnancy Hypertension 2018.

https://doi.org/10.1016/j.preghy.2018.06.012

2.0 Chapter 2: Exploring the role of mitochondrial dysfunction in the pathophysiology of pre-eclampsia.

2.1 Abstract

Background: Uncomplicated pregnancy is associated with an increase in oxidative stress; however, in pregnancies affected by pre-eclampsia there is an exaggerated oxidative stress phenotype with a correspondent deficient antioxidant response. There is circumstantial evidence linking mitochondrial dysfunction as a source of oxidative stress in pre-eclampsia.

Objectives: This study aimed to investigate if mitochondrial dysfunction was evident in pre-eclampsia by measuring superoxide dismutase activity and mitochondrial DNA in maternal blood. This study also investigated if lifestyle and dietary factors impact mitochondrial function and alter the risk of pre-eclampsia.

Study Design: A nested case-control study was performed within the SCOPE Ireland study. Plasma samples were taken at 15 and 20 weeks' gestation from women who subsequently developed pre-eclampsia (n=60) and matched with controls (n=120). SOD activity was determined using a superoxide dismutase assay kit. mtDNA was measured by absolute quantification using real-time PCR. Statistical analysis was performed using GraphPad Prism and SPSS version 22.

Results: There was significant reduction in antioxidant SOD activity at 15 weeks' gestation 1.69 ng/ml ± 0.06 vs 1.94 ng/ml ± 0.06; $p< 0.01$) and an increase in the mean difference in mtDNA [2236 ± 796.0 copies/ml vs -555.3 ± 599.3 copies/ml; $p<0.0065$] (between 15 and 20 weeks' gestation) in cases compared to controls. Lifestyle or dietary factors had no effect on mitochondrial function.

Conclusion: This study confirms a pathogenic role for mitochondrial dysfunction in the pathophysiology of pre-eclampsia. Further research is required in larger diverse populations to explore targeting mitochondrial dysfunction as a potential therapeutic for pre-eclampsia.

2.2 Introduction

Pre-eclampsia is a pregnancy disease that complicates 2-5% of pregnancies worldwide (273). It is characterised by the development of hypertension and proteinuria after 20 weeks' gestation (273). Pre-eclampsia is thought to occur secondary to abnormal placentation in early pregnancy (274) resulting from impaired placental trophoblast invasion and subsequent generation of an ischemic environment (266). Placental ischemia is proposed to increase placental oxidative stress leading to the shedding of syncytiotrophoblast debris into the maternal circulation where it initiates a systemic maternal inflammatory response and subsequent endothelial dysfunction (275).

Oxidative stress is a physiological condition of elevated levels of reactive oxygen species (ROS) which damage cell structure and function. Antioxidant enzymes including superoxide dismutase (SOD) and glutathione peroxidase are components of the body's mechanism for combating oxidative stress. SOD is a powerful antioxidant that catalyses the reaction between two identical molecules of superoxide radical into oxygen or hydrogen peroxide. There is significant evidence that oxidative stress plays a role in the pathophysiology of pre-eclampsia (276, 277). Normal pregnancy is associated with an increase in oxidative stress due to a rise in maternal metabolism and maternal blood flow in the placenta by 10–12 weeks' gestation (57). However, in pregnancies complicated by pre-eclampsia there is an exaggerated oxidative stress phenotype with a correspondent deficient antioxidant response.

Mitochondria are the dominant cellular source of ROS and there is strong evidence that mitochondrial ROS (mROS) play an important role in a variety of physiological

processes including the regulation of cell differentiation, apoptosis, redox cell signalling and inflammation (270, 278, 279). Furthermore, our research has implicated mitochondrial dysfunction as a potential mediator of oxidative stress in pre-eclampsia (279). Cell-free DNA (cf-DNA) has been investigated as a universal diagnostic biomarker for a number of clinical applications, such as prenatal diagnosis and cancer monitoring (280, 281). Circulating cell free DNA is composed of both nuclear and mitochondrial DNA.

Mitochondrial DNA (mtDNA) encodes for 37 genes programmed by the mitochondrial genome (78) and is often used as a biomarker of mitochondrial dysfunction. mtDNA are particularly vulnerable to oxidative damage due to its intimate location in the electron transport chain (ETC) in the mitochondrial matrix and its lack of protective histones (282). More recently, there is emerging evidence suggesting that cell-free mtDNA (cf-mtDNA) is linked to disease progression such as cardiovascular disease (283). Circulating DNA in maternal plasma is mostly of maternal origin (hematopoietic and stromal derived) depending on gestational week and maternal bodyweight. Approximately 5-20% of the circulating DNA is derived from fetal/placental cells (284).

Our research has previously shown an increase in mtDNA in plasma samples at time of disease (TOD) in women with pre-eclampsia (109). Hence the aim of this study was to characterise the role of mitochondrial dysfunction in women with pre-eclampsia compared to uncomplicated pregnancies by assessing levels of antioxidant enzyme superoxide dismutase and mtDNA at earlier time-points in pregnancy. We also examined if lifestyle and dietary factors affected mtDNA levels in pregnancy.

We hypothesised that mitochondrial dysfunction plays a role in the pathogenesis of pre-eclampsia.

2.3 Methods

2.3.1 Study subjects

Subjects were recruited from the SCOPE study Ireland which is an international multicentre prospective cohort study of nulliparous singleton pregnancies aimed to develop a screening test to predict adverse pregnancy outcomes including pre-eclampsia, SGA infants and spontaneous pre-term birth (285, 286). The Clinical Research Ethics Committee, University College Cork, approved the collection and use of samples for research purposes. A nested case-control study within SCOPE Ireland was conducted which included all pre-eclampsia cases in SCOPE Ireland and matched controls with a case-to-control ratio of 1:2.

Pre-eclampsia cases were defined as women with systolic blood pressure ≥ 140mm Hg and/or diastolic blood pressure ≥ 90 mm Hg on at least two occasions 4 hrs apart after 20 weeks' gestation and with proteinuria (24 hour urinary protein ≥ 300mg or urine dipstick protein ≥ +2). Randomly selected controls were taken from healthy pregnant women who had uncomplicated pregnancies which were defined as pregnancies not affected by pre-eclampsia, preterm birth or growth restriction and delivered at >37 weeks. All blood pressure readings were <140 and/or <90 mmHg prior to the onset of labour. These were matched with the cases for maternal age, body mass index (BMI) and gestational age. Both 15 and 20 week samples were taken from the SCOPE study from women who subsequently went onto develop pre-eclampsia (n=60) and controls (n=120). Samples were also taken from a subset of women (n=25) at the time of disease (TOD) with pre-eclampsia.

2.3.2 Superoxide Dismutase enzyme activity

Superoxide dismutase activity was quantified in citrate plasma samples using a superoxide dismutase assay kit (Cayman Chemical) which was used as per manufacturer's instructions. This assay utilizes tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. This assay measures a combination of activity from all three isoforms.

2.3.3 Sample collection and DNA extractions

Plasma samples were collected in BD Heparin Vacutainer tubes, placed on ice, and centrifuged at 2,400g for 10 minutes at 4˚C according to the standardised protocol. Plasma samples were stored at -80˚C until analysis. Samples were analysed in a blinded manner. Total DNA was extracted from 200µl of plasma from both controls and cases respectively with a QIAamp DNA mini kit (Qiagen). DNA was sonicated at 38 kHz ± 10% for 10 minutes to optimise DNA yield.

2.3.4 mtDNA quantification

Mitochondrial DNA was measured by real-time PCR using StepOne Plus Detection system using Taqman assays for mitochondrial DNA (hMitoF5, hMitoR5) (287). Absolute quantification of the concentration of mitochondrial DNA (mtDNA) was determined by standard curve analysis and presented as copies/ml (287, 288).

2.3.5 Maternal lifestyle factors

Women were asked at recruitment (15 ± 1 weeks of gestation) and at their second visit (20 ± 1 weeks of gestation) how many times each week did they carry out exercise that did not result in heavy breathing, which was the SCOPE definition of moderate-intensity exercise. The response was categorised as never, 1-3 times a

week and daily. Similarly, the questionnaire which was completes at both time points asked women to report the frequency in which the consumed fruit and leafy vegetables. Scoring was similar to exercise, where the response was categorised as never, less than five pieces a week, and daily. Multivitamins intake were categorised into never, less than daily and daily.

2.3.6 Statistical analysis

Analysis was performed using GraphPad Prism and SPSS version 22 (SPSS Inc. Chicago, Illinois). Data were presented using median (±Interquartile range [IQR]) and comparisons of data between cases and controls were performed using a non-parametric Mann Whitney U test or Wilcoxon signed rank test as appropriate when data was not normally distributed. Data that was normally distributed were represented as mean (±SEM) and comparisons of data between cases and controls were performed using an unpaired t-test. P values <0.05 were considered as statistically significant. Chi-squared test and the odds ratio (OR) was used to compare categorical variables.

2.4 Results

2.4.1 Patient Characteristics

There were 1,774 participants in the SCOPE Ireland study. 68 (3.8%) women were diagnosed with pre-eclampsia and 60 were included in the nested case-control study with 120 participants selected as controls. The 60 women with pre-eclampsia were composed of 39 women who developed term pre-eclampsia and 21 preterm pre-eclampsia cases. As all cases and controls were matched nulliparous women, there was no significant differences observed between case-controls studies for maternal age, BMI, and gestational age at delivery. There was a significant difference in mean arterial blood pressure (MAP) in controls versus cases at both 15 and 20 weeks' gestation respectively (median [IQR]; 78.0 mmHg [73.33-83.33], n=120, vs median [IQR]; 82.0 mmHg [75.0- 87.66], n=60, p= 0.0015 and media [IQR] 80.41 mmHg [75.3333- 85.0], n=120, vs median [IQR]; 83.5 mmHg [77.5- 89.83] n=60, p= 0.02). There was a significant difference in birthweight in controls compared to cases (3608.93 g ± 411.90 vs 2990.86 g ± 759.24; n=120, n=60; p<0.0001). (Table 2.1).

Table 2.1: Patient Characteristics in the study cohort

	Preterm pre-eclampsia (n=21)	**Term pre-eclampsia (n=39)**	**No pre-eclampsia (n=120)**
Mean Maternal age, years	31	29	29
Mean BMI	25	26	25
Maternal			
Mean Arterial Blood Pressure at 15 weeks	82.33 [74.16-86.33]	81.5 [74.58-88.0]	78 [73.33-83.33]
Mean Arterial Blood Pressure at 20 weeks	81.33 [77.66-89.66]	83 [76.08-87.0]	80.41 [75.33-85.0]
Fetal			
Mean Birth weight, g	2104	3300	3608
Mean gestational age at delivery (weeks)	34	38	40
Perinatal death	1	1	0

Data are presented as mean or Median [IQR]. Mean Arterial blood pressure was calculated as MAP = (2 x diastolic) + systolic/3 and

2.4.2 Evidence of altered plasma SOD activity before pre-eclampsia

There was a statistically significant reduction in antioxidant SOD activity at 15 weeks' gestation between controls and cases (1.94 ng/ml ± 0.06 vs 1.69 ng/ml ± 0.06; n=60, n=119; p< 0.01; 95% CI; 0.04 to 0.45; Figure 2.1A). There was no significant difference in SOD activity at 20 weeks' gestation between controls and cases, (0.82 ng/ml ± 0.02 vs 0.77 ng/ml ± 0.03; n=119 p=0.21; 95% CI; -0.02 to 0.12; Figure 2.1B).

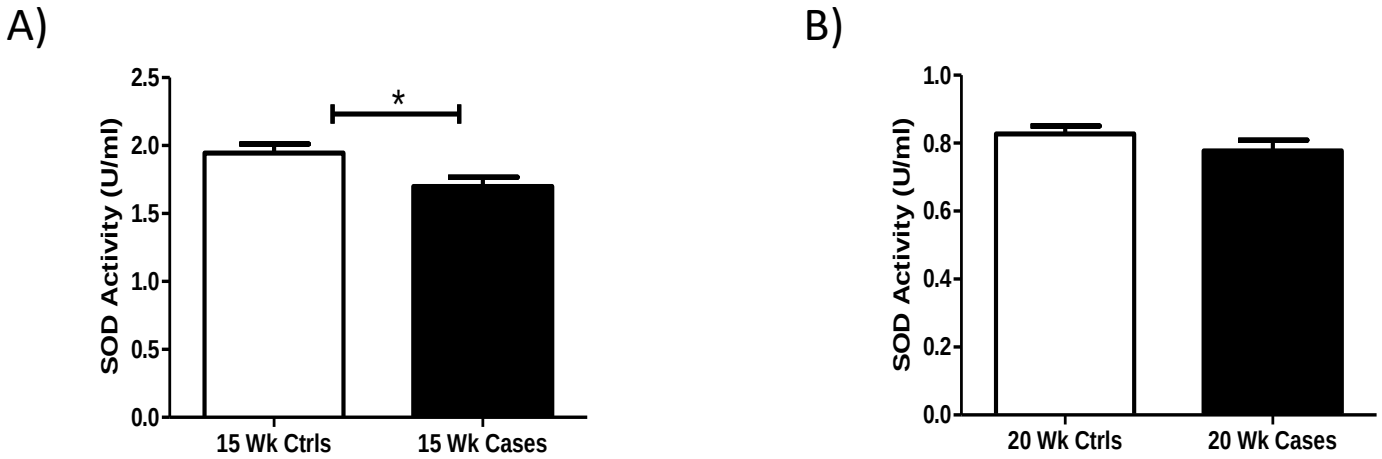

Figure 2.1: A) SOD activity (U/ml) was significantly reduced at 15 weeks in cases compared to healthy controls ($P<0.01$). B) SOD activity (U/ml) at 20 weeks was reduced in cases compared to healthy controls. Data are expressed as mean ± SEM.

2.4.3 Increased ΔmtDNA levels was evident between 15 and 20 weeks' gestation before the onset of clinical symptoms of pre-eclampsia

There was no significant difference in the amount of total DNA between controls and cases at both 15 and 20 weeks' gestation (15 weeks: 7.06 ng/ml ± 4.08 vs 7.70 ng/ml ± 5.43, n =60 n=120, p=0.38; 20 weeks 6.71 ± 3.26 vs 7.15 ± 3.44, n= 60 n=120, p=0.40; Figure 2.2A). There was no significant difference in mtDNA at 15 weeks' gestation between controls and cases (median [IQR]: 2832.96 copies/ml [1711.17-5002.82] vs 2337.32 copies/ml [1357.11-5328.35], n=58-117; p< 0.3381, Figure 2.2B). Similarly, there was no significant difference in mtDNA at 20 weeks' gestation between controls and cases and (median [IQR]: 2885.57 copies/ml [1914.54-4834.12] vs 3307.7 copies/ml [1544.49-7396.92], n=58-117; p<0.7873, Figure 2.2C). As pregnancy progressed the amount of mtDNA significantly increased in cases and controls (median [IQR]: 2337.32 copies/ml [1357.11-5328.35], 3307.7 copies/ml [1544.49-7396.92] and 6449.8 copies/ml [477.54-11145.9] n=58 and n=22.p< 0.0001, and median [IQR]: 2855.41 copies/ml [1740.19-5322.08], 2900.95 copies/ml [1958.36-5055.16], 5983.88 copies/ml [3209.67-16901.5] n-117 and n=23. P=0.009, Figure 2.2D) at 15 weeks', 20 weeks' and TOD respectively.

However, the mean difference in mtDNA (ΔmtDNA, difference in mtDNA copy number between 20 weeks and 15 weeks) was calculated for each individual patient in both cases and controls. The mean difference in mtDNA between 20 weeks and 15 weeks gestation was then calculated for control group and case group. These values were then compared between cases and controls and found that there was significantly higher ΔmtDNA in cases compared with controls between 15 weeks'

and 20 weeks' gestation (2236 ± 796.0 copies/ml vs -555.3 ± 599.3 copies/ml mtDNA concentration in plasma, p=0.0065; Figure 2.2E).

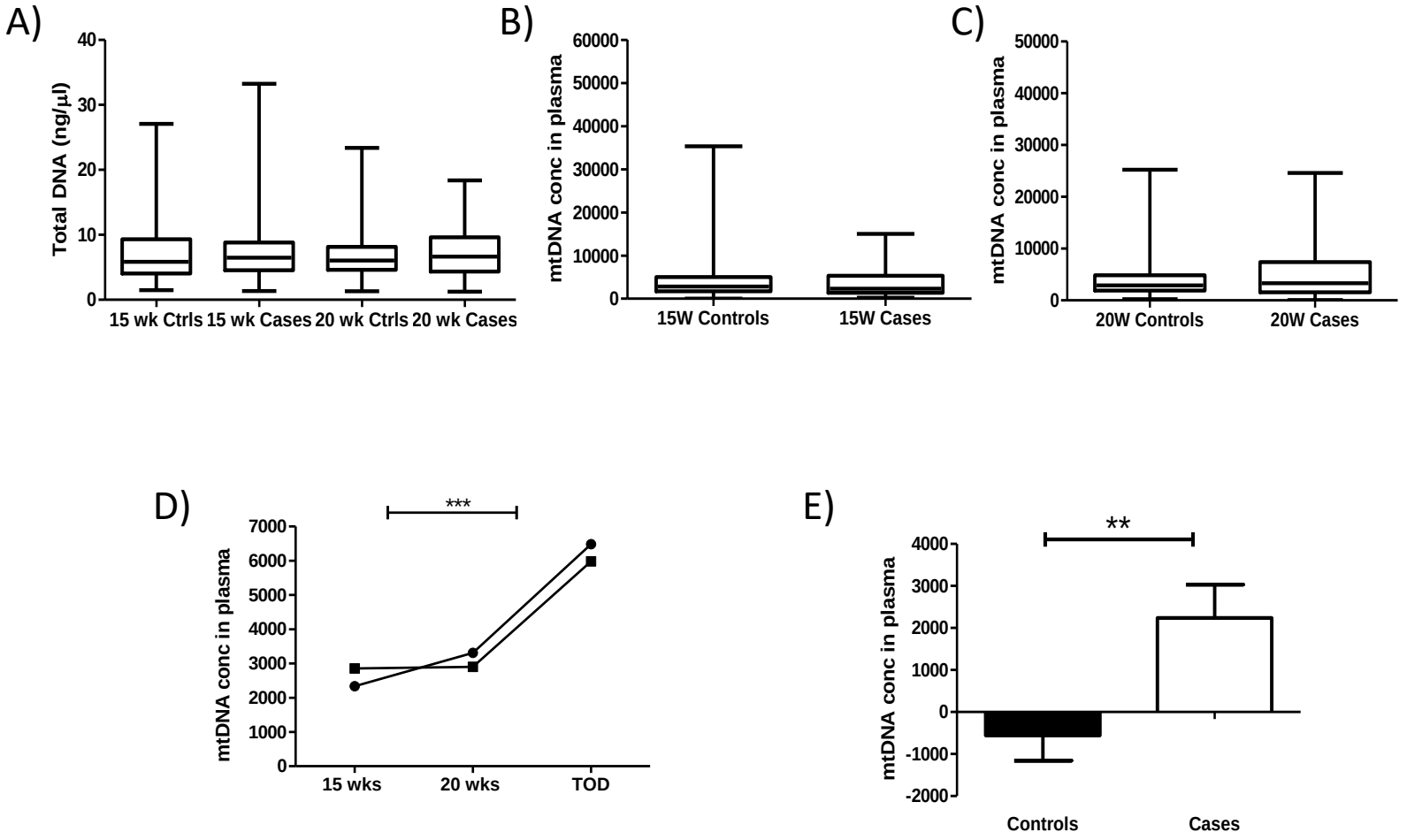
A)
Total DNA (ng/µl)
40
30
20
10
0
15 wk Ctrls
15 wk Cases
20 wk Ctrls
20 wk Cases
B)
mtDNA conc in plasma
60000
50000
40000
30000
20000
10000
0
15W Controls
15W Cases
C)
mtDNA conc in plasma
50000
40000
30000
20000
10000
0
20W Controls
20W Cases
D)
***
mtDNA conc in plasma
7000
6000
5000
4000
3000
2000
1000
0
15 wks
20 wks
TOD
E)
**
mtDNA conc in plasma
4000
3000
2000
1000
0
-1000
-2000
Controls
Cases

Figure 2.2: A) Total DNA in maternal plasma in controls and cases showed no significant difference. B) mtDNA at 15 weeks showed no significant difference in controls when compared to cases C) mtDNA at 20 weeks was higher in controls compared to cases but not statistically significant D) Mitochondrial dysfunction is significantly increased as gestation progresses in pre-eclampsia and uncomplicated pregnancies ($p<0.001$ and $p=0.0009$) respectively ●=cases, ■=control. Data represented as the median; [IQR]. E) Significant increase in the mean difference in mtDNA concentration between 15 and 20 weeks' gestation in cases compared to controls ($P<0.01$). Data represented as the mean; [SEM]

2.4.4 Lifestyle and nutritional factors; effect of these factors on mtDNA

Moderate exercise had no significant impact on the amount of mtDNA in controls compared with cases at 15 or 20 weeks' gestation OR 1.00; [CI 1.00-1.00 vs 1.00 CI [1.00-1.00] respectively (Table 2.2A). Similarly, when assessing dietary factors such as leafy vegetable intake, fruit intake and multivitamin consumption, there was no effect on the amount of mtDNA at 15 or 20 weeks' gestation in controls compared with cases (Table 2.3A, 3B, 3C).

Table 2.2: The effect of lifestyle factors (Exercise) on mtDNA in cases compared to controls at 15 and 20 weeks' gestation

Moderate exercise activity	**N=58**	**Case (mtDNA copy number/ml)**	**N=117**	**Control (mtDNA (copy number/ml**	**OR**	**(95% CI)**
Never exercised (15 weeks' gestation)	19	1631.02 (815.92-3445.15)	23	2817.51 (1856.43-5602.91)	1.00	1.00-1.00
More than Once a week (15 weeks' gestation)	31	2742.43 (1510.48-5328.35)	60	2676.89 (1669.87-3734.28)		
Daily (15 weeks' gestation)	8	3928.08 (934.06-11509.39)	34	3070.21 (1912.75-5322.08)		
Never exercised (20 weeks' gestation)	12	3242 (1367.75-9276.86)	20	2649.15 (1849.56-4454.55)	1.00	1.00-1.00
More than once a week (20 weeks' gestation)	37	3249.79 (1493.10-7562.22)	67	2847.66 (1758.46-4248.59)		
Daily (20 weeks' gestation)	9	4198.29 (1632.68-5398.94)	30	2790.07 (1998.00-5385.22)		

Table 2.3 (A): The effect of dietary factors (Fruit intake) on mtDNA in cases compared to controls at 15 and 20 weeks' gestation

High fruit intake	**N=58**	**Case (mtDNA copy number/ml)**	**N=117**	**Control (mtDNA copy number/ml)**	**OR**	**(95% CI)**
Never (15 weeks' gestation)	7	2158.15 (1357.11-3119.63)	9	1856.43 (617.47-9759.18)	1.00	1.00-1.00
<6 times a week (15 weeks' gestation)	11	3445.15 (1140.94-6661.19)	25	2617.49 (1683.27-4005.63)		
>5 a day (15 weeks' gestation)	40	2466.71 (1339.17-4526.35)	83	2829.32 (1868.68-4786.10)		
Never (20 weeks' gestation)	0		1	6802.61	1.00	1.00-1.00
<6 times a week (20 weeks' gestation)	6	2650.57 (1063.15-6136.60)	16	2384.71 (1471.25-5954.11)		
>5 a day (20 weeks' gestation)	52	3344.25 (1548.32-7395.89)	108	2856.83 (1980.26-4711.28)		

Table 2.3 (B): The effect of dietary factors (leafy vegetable intake) on mtDNA in cases compared to controls at 15 and 20 weeks' gestation

Leafy vegetable intake	**N=58**	**Case (mtDNA copy number/ml)**	**N=117**	**Control (mtDNA (copy number/ml**	**OR**	**(95% CI)**
Never (15 weeks' gestation)	22	2064.62 (1443.17-5873.68)	49	2862.43 (1964.75-5110.42)	1.00	1.00-1.00
<6 times a week (15 weeks' gestation)	27	2133.39 (1140.94-4068.91)	42	2547.10 (1644.76-4838.29)		
>5 a day (15 weeks' gestation)	9	3119.63 (1677.58-5812.97)	26	2676.89 (1184.76-3950.15)		
Never (20 weeks' gestation)	1	4198.29 (4198.29-4198.29)	3	1846.20 (1108.22-)	1.00	1.00-1.00
<6 times a week (20 weeks' gestation)	23	3104.99 (1366.01-7396.91)	46	2596.07 (1636.75-4126.70)		
>5 a day (20 weeks' gestation)	34	3315.32 (1518.79-6499.72)	68	3067.12 (2151.85-5347.62)		

Table 2.3 (C): The effect of dietary factors (Multivitamin intake) on mtDNA in cases compared to controls at 15 and 20 weeks' gestation

Multivitamin intake	**N=58**	**Case (mtDNA copy number/ml)**	**N=117**	**Control (mtDNA (copy number/ml**	**OR**	**(95% CI)**
No (15 weeks' gestation)	44	2206.55 (1186.20-4709.44)	71	2617.49 (1856.43-4778.04)	1.00	1-1.00
Daily (15 weeks' gestation)	14	2745.61 (1504.21-6598.51)	39	2850.34 (1626.85-5111.71)		
Less than daily (15 weeks' gestation)	0		7	3518.35 (1856.43-4315.26)		
No (20 weeks' gestation)	38	4032.60 (1744.82-7789.96)	57	2777.50 (1769.47-5184.31)	1.00	1-1.00
Less than daily (20 weeks' gestation)	4	1374.57 (332.18-5948.15)	15	3647.68 (2278.63-4711.28)		
Daily (20 weeks' gestation)	16	2384.71 (1471.25-5954.11)	45	2754.95 (1822.09-4421.01)		

2.5 Discussion

Mitochondrial dysfunction is a pathogenic mediator of oxidative stress in pre-eclampsia with elevated mitochondrial lipid peroxidation and increased vulnerability to oxidation evident in placental mitochondria in pregnancies complicated by pre-eclampsia (104). In this study we showed a significant reduction in antioxidant SOD activity at 15 weeks' gestation and a corresponding increase in ΔmtDNA (between 15 and 20 weeks' gestation) in cases compared to controls.

Oxidative stress results from an imbalance in the production of ROS and the responsive antioxidant levels. There is a vast amount of evidence for antioxidant decline and elevation of ROS in pre-eclampsia (277, 289). SOD is the first barrier and antioxidant defence against ROS and its activity is increased in the placenta of a normal pregnancy (62), while SOD activity in placental tissue from women with pre-eclampsia is decreased (290). In our study, we showed lower levels of SOD activity at 15 weeks' gestation in cases compared to controls. This correlates with previous work which showed lower levels of SOD at both 10-14 and 20-24 weeks' gestation respectively in pre-eclampsia (291). Similarly, the levels of maternal erythrocyte SOD were also lower in the second half of pregnancy in pre-eclampsia when compared with normotensive pregnancies (277).

Mitochondrial DNA is correlated with the number and size of the mitochondria (292), furthermore mtDNA are particularly susceptible to oxidative damage. While the origin of cf-mtDNA is difficult to phenotype, the quantitative assessment of cf-mtDNA may permit the evaluation of mitochondrial dysfunction in pre-eclampsia. There have been a number of studies that suggest mitochondrial abundance may be associated with placental insufficiency and pre-eclampsia (268, 272, 293, 294). Our

research previously showed evidence of increased mtDNA at time of disease in women with pre-eclampsia, furthermore, we expanded this work and provided additional evidence of mitochondrial dysfunction by demonstrating increased mitochondrial-specific ROS and reduced oxygen consumption (109). In this current study, while there was no significant difference in mtDNA copy number at both 15 and 20 weeks' gestation respectively in cases compared with controls, we reported a significant increase in ΔmtDNA copy number between 15 and 20 weeks' gestation in cases compared with controls.

Given the critical role of SOD antioxidants in mediating oxidative damage provoked by exaggerated superoxide generation, the compromised antioxidant defence evident at 15 weeks' gestation in cases in our study group may be partly responsible for increased vulnerability of mtDNA to damage as evident by the increase in ΔmtDNA between 15-20 weeks' gestation. Previous work in retinal endothelial cells overexpressing SOD2 (295) and in SOD2-depleted chrondrocytes (296) established an essential protective role for this enzyme in preventing mtDNA damage. Furthermore, we showed that mtDNA copy number increases as pregnancy progresses in women with pre-eclampsia and we hypothesise that the initial insult to mitochondrial antioxidant function seen early in pregnancy (15 weeks) could be exacerbated later in pregnancy resulting in a more significant increase in mtDNA copy number in pre-eclampsia as we previously described.

The strength of our data compared to previous work in this area is attributable to the longitudinal examination of mtDNA through gestation, whereas previous studies have focused on mtDNA quantitation in the third trimester of pregnancy. This work correlates with previous studies, where mtDNA copy number was assessed in a case-control study and reported that the odds of pre-eclampsia were positively associated

with increased maternal blood mtDNA copy number (268). These findings strongly suggest that altered mitochondrial function is evident very early in the development of pre-eclampsia. This study was performed in the Irish SCOPE cohort and while we provide evidence of mitochondrial dysfunction in pre-eclampsia, further larger studies in different populations are warranted.

Lifestyle interventions such as healthy diet (fruit and vegetable consumption) and exercise have been examined in mitochondrial diseases (297, 298) given their physiological effect on mitochondrial function. In recent years, exercise has been intensively examined in relation to reducing risk of pre-eclampsia (299, 300). The next step in this study was to assess whether lifestyle and nutritional factors had an effect on the amount of mtDNA in controls and cases. However, we showed that exercise had no significant difference on mtDNA copy number during pregnancy in controls or cases. Similarly, a previous study assessed exercise and mtDNA copy number in controls and pre-eclampsia cases and showed no association between exercise and mtDNA copy number (268).

Diet has been suggested to play a potential role in the management of pre-eclampsia (301). In our study we found no association between fruit and vegetable intake on mtDNA copy number in control and cases. Similarly, Clausen et al, showed no association between pregnancies affected by pre-eclampsia and healthy pregnancies when investigating meat, fish, vegetables and fruit intake (302). Finally, we investigated multivitamin intake and its association with mtDNA copy number in control and cases and found no association. Both Vitamin C and E have been extensively studied as antioxidant therapeutic options in pre-eclampsia (187, 303), however the results were largely disappointing. This may have occurred as these exogenous antioxidant vitamins do not penetrate the intracellular source of ROS, the

mitochondria, and are sequestered in the cytosol. Therefore, we propose a mitochondrial-targeted antioxidant may represent a more promising clinically effective treatment strategy for pre-eclampsia.

2.6 Conclusion

In this study, we provide evidence that in early gestation there is a significant reduction in mitochondrial antioxidant SOD activity in women who developed pre-eclampsia. Furthermore, there is a significant increase in ΔmtDNA levels between 15 and 20 weeks' in women who subsequently went on to develop pre-eclampsia. Our findings support a pathogenic role for mitochondrial dysfunction in the pathophysiology of pre-eclampsia. Finally, we found no effect of either lifestyle or dietary factors in mediating mitochondrial dysfunction in this study cohort, highlighting the potential need for the development for mitochondrial targeted antioxidants as potential therapeutic targets to treat pre-eclampsia.

Chapter 3: Activation of a TLR9 mediated innate immune response in pre-eclampsia

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3.0 Chapter 3: Activation of a TLR9 mediated innate immune response in pre-eclampsia.

3.1 Abstract

Introduction: Pre-eclampsia is a multisystemic disorder leading to the development of a placental ischemic microenvironment with a resultant increase in oxidative stress. There is evidence that mitochondrial dysfunction and the innate immune system both play a role in the pathophysiology of this disease. Mitochondrial DAMPs such as mtDNA bind specific pattern recognition receptors such as Toll-like receptor 9 (TLR9) on the endosomal surface of immune cells, in particular neutrophils, subsequently activating them and triggering an innate response.

Objective: We hypothesised that the exaggerated innate immune response seen in preeclampsia is provoked by dysfunctional mitochondria.

Results: Here we provide evidence that TLR9 activity is significantly increased at time of disease in women with pre-eclampsia. Furthermore, we show activation of neutrophil markers, calprotectin, myeloperoxidase (MPO), and IL-8 are significantly increased at time of disease compared to uncomplicated pregnancies.

Conclusion: This research supports a potential role of TLR9 activation of an innate immune response evident in pre-eclampsia which may possibly be initially triggered by dysfunctional mitochondria.

3.2 Introduction

Pre-eclampsia is multifactorial disorder of pregnancy that is defined by the onset of hypertension and proteinuria after 20 weeks' gestation. One of the most established characteristics of this disorder is the inability of the trophoblasts to invade the maternal uterine decidual arteries, resulting in poor placentation. Production of placental anti-angiogenic factors such as soluble fms-related tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng) have been extensively researched in pre-eclampsia (304, 305). More recently, the cardiovascular system and its role in the development of pre-eclampsia is being explored (306). Other pathological characteristics of pre-eclampsia include placental and systemic oxidative stress and dysfunction of the maternal vasculature (259, 307). In normal pregnancies there is evidence of a controlled systemic inflammatory response where cytokines promote the infiltration of the spiral arteries by invading trophoblast cells (308). This controlled inflammatory response becomes dysregulated in pre-eclampsia resulting in abnormal activation of monocytes, neutrophils and the endothelium causing maternal inflammation (309).

There is overwhelming evidence that oxidative stress plays a key role in the pathophysiology of pre-eclampsia (55, 276). In turn oxidative stress, as a result of a placental ischemic microenvironment, releases reactive oxygen species into the maternal circulation, which can provoke a systemic inflammatory response (275). The innate immune system acts as both a protector and effector during pregnancy. The innate system encompasses neutrophils, dendritic cells, natural killer cells and macrophages and these immune responders are activated to protect the mother from pathogens. Toll-like receptors (TLRs) are a family of type I transmembrane pattern

recognition receptors (PRRs) that identify invading pathogens or endogenous damage signals and instigate an innate immune response.

TLR9 can detect conserved sequences known as pathogen-associated molecular patterns (PAMPs) and also specifically respond to endogenous molecular structures known as damage-associated molecular patterns (DAMPs) via unmethylated CpG dinucleotide motifs as evident on mitochondrial DNA (270). Activation of endosomal TLR9 involves an intracytoplasmic signalling cascade that leads to the up-regulation of pro-inflammatory transcription factors and subsequent liberation of pro-inflammatory cytokines (134). There is evidence of increased TLR9 expression in both placental tissue and peripheral blood mononuclear cells (PBMC) from patients with pre-eclampsia compared with controls (140, 310).

Neutrophils are short-lived effector cells of the innate immune system. These immune cells are activated in women during pregnancy and are further stimulated in pre-eclampsia (311). Histopathological evidence has shown extensive infiltration of neutrophils in the systemic vasculature of women with pre-eclampsia compared to controls (312, 313). Neutrophils express a number of TLRs on their surface including TLR9 (314). Mitochondrial DNA (mtDNA) has been shown to activate neutrophils via a TLR9 signalling cascade, which elicits a neutrophil pro-inflammatory phenotype (136, 141, 315). Neutrophil activation results in the secretion of a number of markers including reactive oxygen species (ROS), matrix metalloproteinase-8 (MMP-8), calprotectin, myeloperoxidase (MPO) and the pro-inflammatory cytokine IL-8 (316).

Our research has previously provided evidence of a role for mitochondrial dysfunction in the pathophysiology of pre-eclampsia with an increase in ΔmtDNA

evident between 15 and 20 weeks in women with pre-eclampsia (317). Interestingly we also showed a significant increase in mtDNA at time of disease (TOD) in women with pre-eclampsia compared to controls (109). The aim of this study was to investigate if the increase in mtDNA we previously reported in pre-eclampsia triggers activation of TLR9 signalling cascade. We also wanted to determine if a subsequent neutrophil pro-inflammatory phenotype was elicited in pre-eclampsia compared to controls by measuring a number of neutrophil activation markers longitudinally in pregnancy. We hypothesised that dysfunctional mitochondria provokes an exaggerated innate immune response in pre-eclampsia.

3.3 Materials and Methods

3.3.1 Study subjects

Subjects were recruited from the SCOPE study Ireland which is an international multicentre prospective cohort study of nulliparous singleton pregnancies aimed to develop a screening test to predict adverse pregnancy outcomes including pre-eclampsia, SGA infants and spontaneous pre-term birth (285, 286). A nested case-control study within SCOPE Ireland was conducted which included all pre-eclampsia cases in SCOPE Ireland and matched controls with a case-to-control ratio of 1:2. Pre-eclampsia cases was defined as a systolic blood pressure ≥ 140mm Hg and/or diastolic blood pressure ≥ 90 mm Hg on at least two occasions 4 hrs apart after 20 weeks' gestation and with proteinuria (24 hour urinary protein ≥ 300mg or urine dipstick protein ≥ +2). Randomly selected controls were taken from healthy pregnant women who had uncomplicated pregnancies which were defined as pregnancies not affected by pre-eclampsia, preterm birth or growth restriction and delivered at >37 weeks. All blood pressure readings were <140 and/or <90 mmHg prior to the onset of labour. These were matched with the cases for maternal age, body mass index (BMI) and gestational age.

Both 15 and 20 week samples were taken from the SCOPE study from women who subsequently went onto develop pre-eclampsia (n=60) and controls (n=120). Samples were also taken from a subset of women (n=25) at the time of disease (TOD) with pre-eclampsia and matched controls. The 60 women with pre-eclampsia were composed of 39 women who developed term pre-eclampsia and 21 preterm pre-eclampsia cases. The SCOPE study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all the procedures were approved by

the Clinical Research Ethics Committee of the Cork Teaching (EMC5(10)05/02/08), and all women provided written informed consent.

3.3.2 Sample collection

Plasma samples were collected in BD Heparin Vacutainer tubes, placed on ice, and centrifuged at 2,400g for 10 minutes at 4˚C according to a standardised protocol. Plasma samples were stored at -80˚C until analysis.

3.3.3 TLR-9 Activity

TLR9 ligand activity was monitored with HEK-blue TLR9 Reporter Cell assay (InvivoGen). All experiments were performed using a cell density of 50,000 cells in a 96 well plate. Cells were initially seeded and left overnight prior to treatment for 24 hrs with 3% plasma taken at 15 and 20 weeks' gestation (cases, n=60, controls, n=120) and TOD (cases, n=25, controls, n=25). The supernatant was incubated with Quanti-Blue detection medium (InvivoGen) and the activity was read on a Varioskan Flash plate reader (Thermo Scientific) at a wavelength of 630 nm.

3.3.4 Calprotectin analysis

Plasma calprotectin (S100A8/S100A9) concentrations were measured by enzyme-linked immunosorbent assay (ELISA) using human S100A8/S100A9 Quantikine kit. Heparin plasma samples from all time-points were initially diluted a 100-fold in assay buffer and then directly added to a pre-coated plate. The ELISA was performed as per manufacturers' instructions.

3.3.5 Myeloperoxidase, MMP8, IL-8 analysis

Myeloperoxidase (MPO), Matrix metalloproteinases-8 (MMP8) and interleukin-8 (IL-8) concentrations were measured respectively at all time-points by individual ELISA DuoSet kits (R&D SYSTEMS. USA & Canada). ELISA was carried out as per manufacturer's instructions.

3.3.6 Statistical analysis

Analysis was performed using GraphPad Prism. Data were presented using median (±Interquartile range [IQR]) and comparisons of data between cases and controls were performed using a non-parametric Mann Whitney U test or Wilcoxon signed rank test as appropriate when data was not normally distributed. Data that was normally distributed were represented as mean (±SEM) and comparisons of data between cases and controls were performed using an unpaired t-test. P values <0.05 were considered as statistically significant.

3.4 Results

3.4.1 Determination of TLR9 activity and downstream markers of neutrophil activation at 15 weeks' gestation

To determine if TLR9 was activated by circulating mediators present in pre-eclampsia, HEK-TLR9 cells were treated with 3% plasma. The level of neutrophil activation markers, calprotectin, MPO, MMP8 and IL-8 were also examined. Firstly, there was no significant increase in TLR9 activity at 15 weeks' gestation in cases compared to controls (0.28 ± 0.02 v 0.2 ± 0.02; P=0.99) (Figure 3.1a). We subsequently measured a number of well described markers of neutrophil activation in both study groups at 15 weeks' gestation. There was no significant increase in calprotectin (1319 ng/ml ± 87.96, v 1198 ng/ml ± 56.76; P= 0.23) (Figure 3.1b) or myeloperoxidase (Median [IQR]: 6.72 ng/ml [2.20 - 13.13] v 4.83 ng/ml [2.11 - 8.41]; P= 0.14) between cases and controls (Figure 3.1c). There was also no significant difference in MMP8 (1041.27 pg/ml ± 42.96 v 1075.62 pg/ml ± 88.24; P= 0.69) (Figure 3.1d) or the pro-inflammatory cytokine IL-8 (median [IQR]: 8.99 [3.32-30.70] v 9.32 [2.18-29.85]; P=0.95) between cases and controls at 15 weeks' gestation (Figure 3.1e).

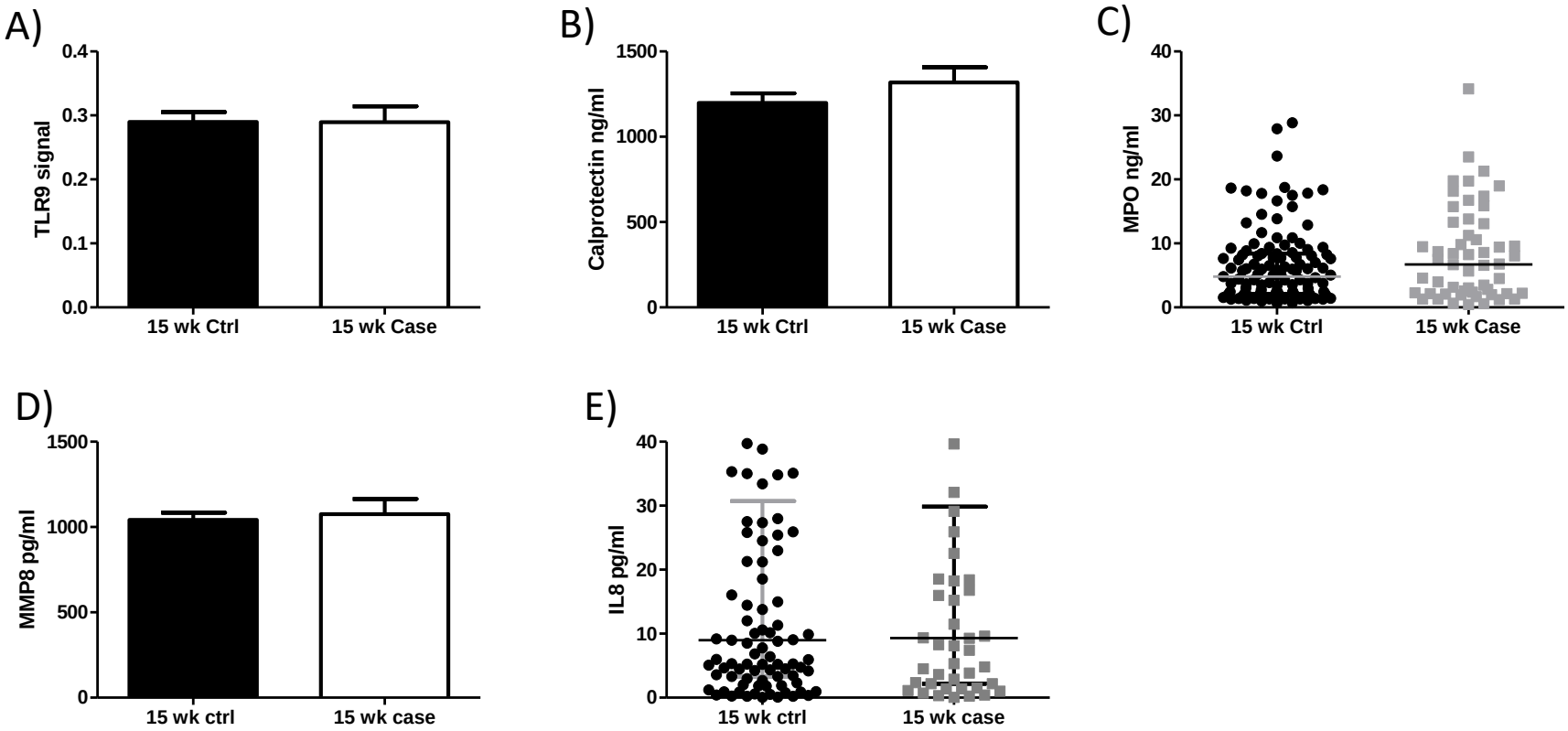


Figure 3.1: TLR9 activity and downstream markers of neutrophil activation did not alter at 15 weeks' gestation. There was no significant increase in TLR9 activity (a) or neutrophil activation markers Calprotectin (b), MPO, (c) MMP-8 (d) and IL-8 (e) at 15 weeks' gestation between cases and controls.

3.4.2 Determination of TLR9 activity and downstream markers of neutrophil activation at 20 weeks' gestation

There was no significant increase in TLR9 activity at 20 weeks' gestation in cases compared to controls (0.29 ± 0.01, v 0.30 ± 0.02; P=0.88) (Figure 3.2a). While there was no change in the levels of calprotectin between cases and controls at 20 weeks' gestation (1155 ng/ml ± 68.45 v 1234 ng/ml ± 61.10; P=0.42) (Figure 3.2b), there was a significant increase in myeloperoxidase in cases compared to controls (Median [IQR]: 5.02 ng/ml [2.36-9.08] v 7.07 ng/ml [2.74-17.24]; P= 0.02) (Figure 3.2c). There was no significant change in the levels of MMP8 between cases and controls (1116.73 pg/ml ± 48.24 v 1053.74 pg/ml ± 54.05; P= 0.41) (Figure 3.2d). There was no significant increase in IL-8 in cases compared to controls at 20 weeks' gestation (Median [IQR]: 7.95 pg/ml [2.52-30.31] v 13.49 pg/ml [3.66-46.66]; P=0.14) (Figure 3.2e).

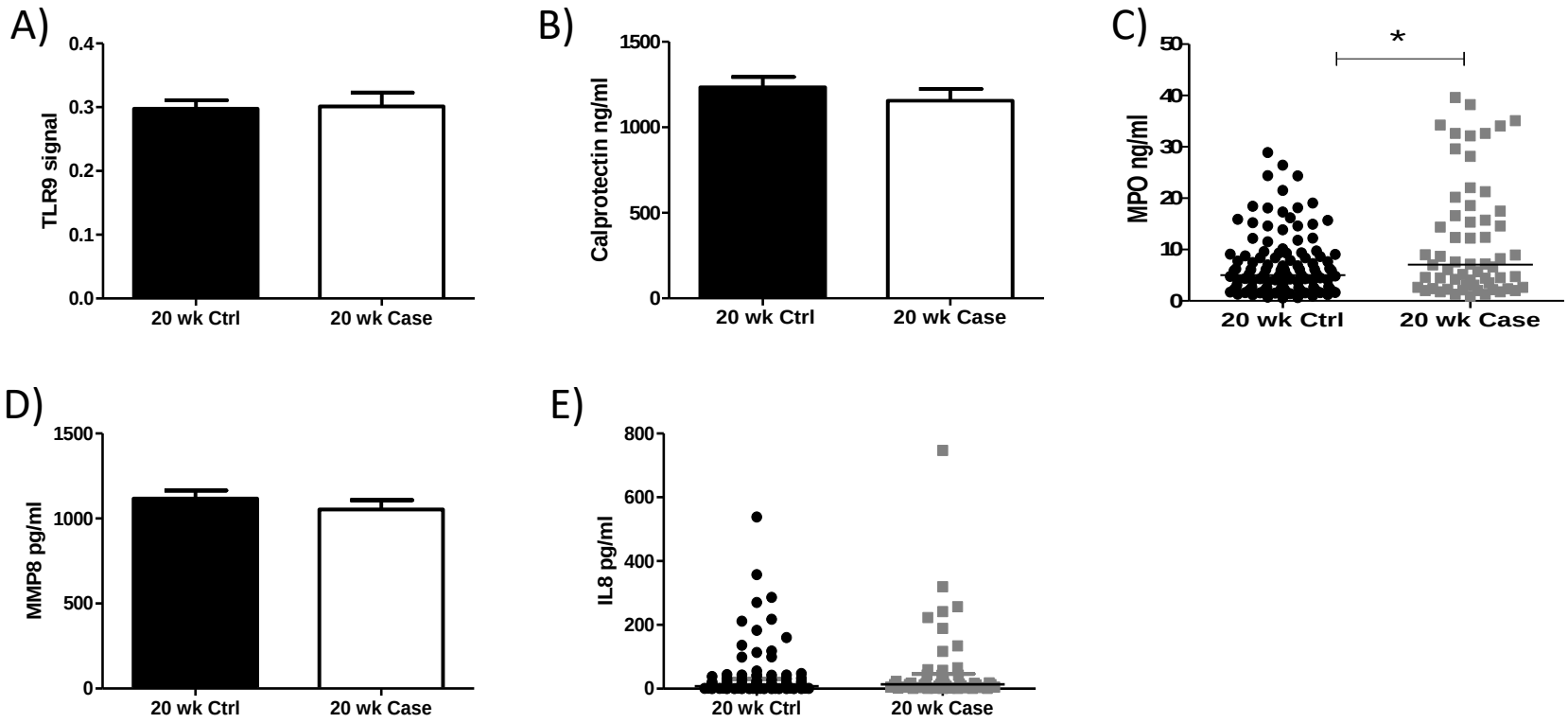


Figure 3.2: TLR9 activity and downstream markers of neutrophil activation did not alter at 20 weeks' gestation. There was no significant increase in TLR9 activity (a) or neutrophil activation markers Calprotectin (b), MMP-8 (d) and IL-8 (e) at 20 weeks' gestation between cases and controls. MPO expression (c) was significantly increased in cases compared with controls (P= 0.02).

3.4.3 Activation of TLR9 activity provokes a neutrophil-derived pro-inflammatory phenotype at time of disease in pre-eclampsia

There was a significant increase in TLR9 activity at TOD in pre-eclampsia cases compared to controls (0.29 ± 0.01 v 0.35 ± 0.01; P=0.01) (Figure 3.3a). Subsequently, there was also a significant increase in both calprotectin (1946.55 ng/ml ± 155.08 v 1516.45 ng/ml ± 145.84; P= 0.04) (Figure 3.3b) and myeloperoxidase (Median [IQR]: 8.33 ng/ml [5.70 - 14.20] v 4.52 ng/ml [3.53 - 8.73]; P= 0.01) (Figure 3.3c) respectively at TOD in cases compared to controls. There was no significant increase in MMP-8 in cases when compared with controls at time of TOD, (842.92 pg/ml ± 87.07 v 1140.64 pg/ml ± 144.77; P= 0.08) (Figure 3.3d). Finally, there was a significant increase in the pro-inflammatory cytokine IL-8 (Median [IQR]: 6.13pg/ml [1.18 - 28.68] v 24.63 pg/ml [5.37 -71.20]; P=0.01) at TOD in cases compared to controls (Figure 3.3e).

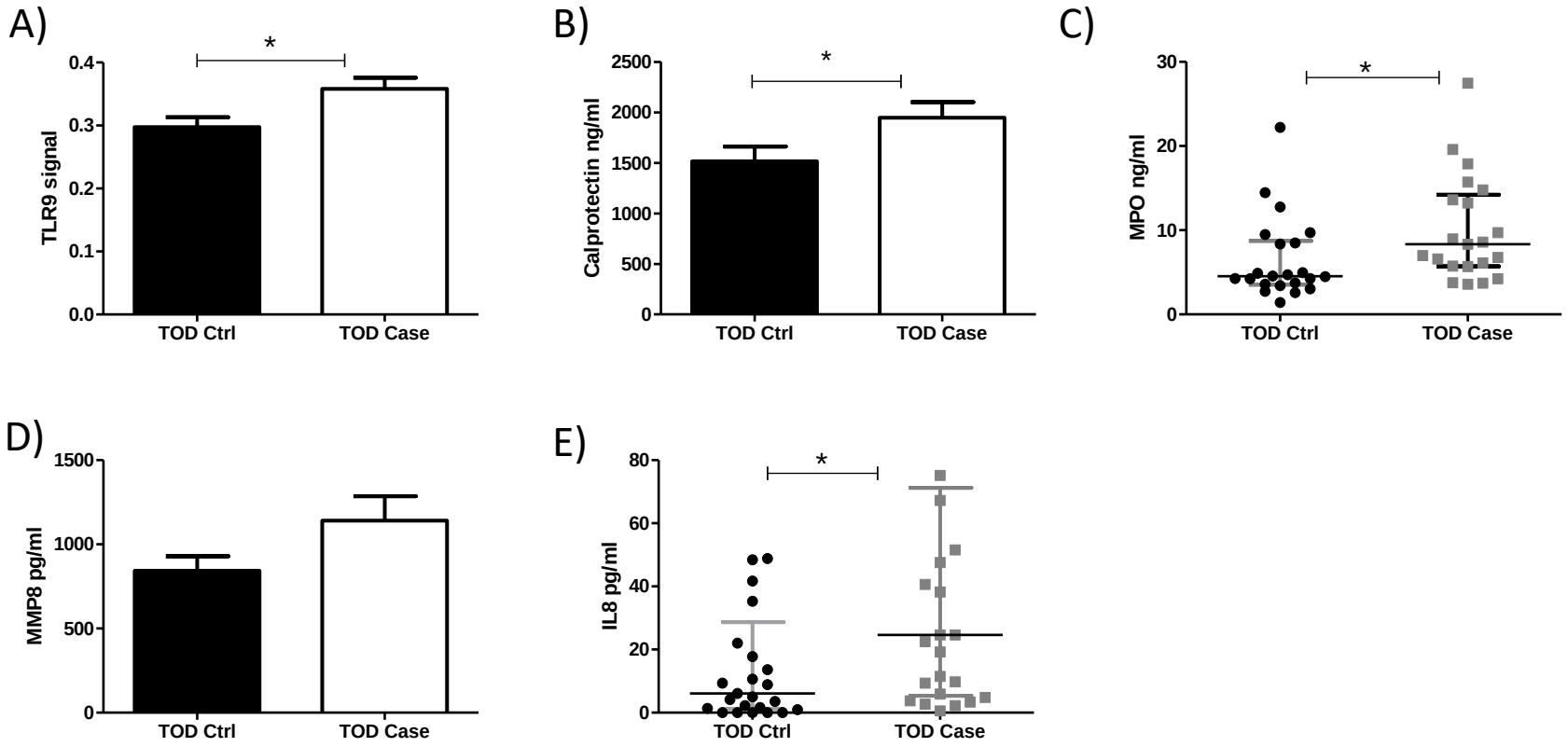


Figure 3.3: Circulating plasma mediators activate a TRL9-mediated innate immune response in pre-eclampsia at TOD. TLR-9 activity (a), Calprotectin (b), MPO (c), and IL-8 (e) were significantly increased at time of disease in cases compared to controls (P=0.01). There was no significant increase in MMP-8 (d) at TOD in cases in comparison to controls.

3.4.4 Evidence of elevated innate immune response across gestation in pre-eclampsia

We investigated if the neutrophil-mediated innate immune response were altered longitudinally in pregnancy. Initially, there was no difference in TLR9 activity across gestation in controls. (Figure 3.4a). Similarly, there was no significant change in calprotectin, MPO and IL-8 expression in controls (Figure 3.4b, c and e). MMP-8 was significantly decreased in uncomplicated pregnancies (Figure 3.4d).

In cases complicated with pre-eclampsia, both TLR9 and calprotectin were significantly increased across gestation (0.28 ± 0.02, 0.30 ±0.02, 0.37 ±0.02; P=0.01) and (1318.69 ng/ml ± 87.95, 1233.77 ng/ml ± 68.45, 1946.55 ng/ml ± 145.84; P = 0.0001) respectively. (Figure 3.5a and b). There was no significant increase in MPO, (6.72 ng/ml [2.20-13.13], 7.09 ng/ml [2.74-17.24], 8.33 ng/ml [5.70-14.20]; P= 0.14), MMP-8 (1075.62 pg/ml ± 88.24, 1053.74 pg/ml ± 54.05, 1140.64 pg/ml ± 87.08; P= 0.82) or IL-8 (9.32 pg/ml [2.18-29.85], 13.49 pg/ml [3.66-46.66], and 24.63 pg/ml [5.37-71.20]; P= 0.13) across gestation in pre-eclampsia cases (Figure 3.5c, 3.5d and 3.5e).

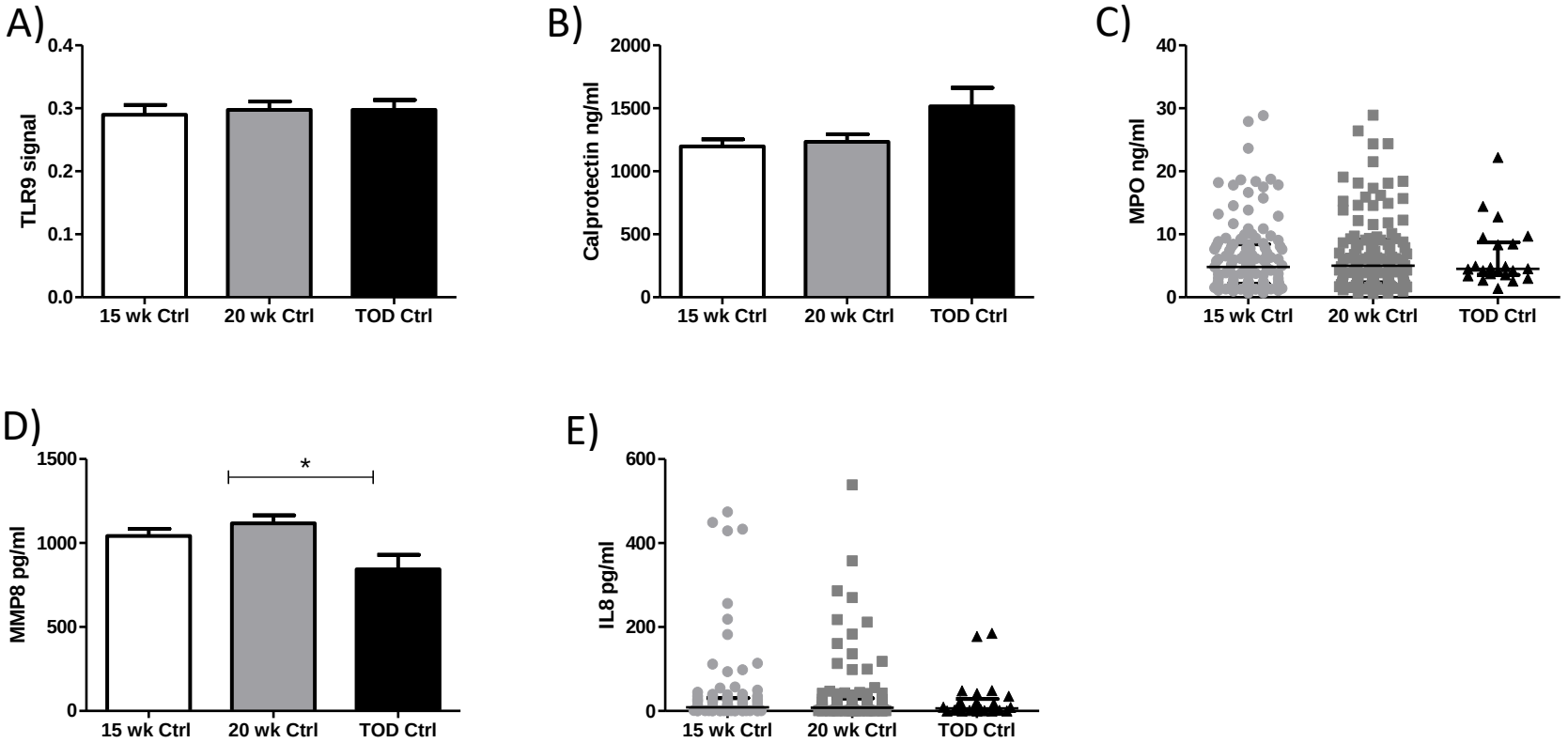


Figure 3.4: Normal pregnancy did not activate a TLR9 mediated innate immune response. There was no significant increase in TLR9 activity (a), Calprotectin (b), MPO (c) in healthy control pregnancies across gestation. MMP8 (d) was significantly reduced at term pregnancies (P= 0.01), while IL-8 (e) showed no significant difference across gestation in healthy control pregnancies

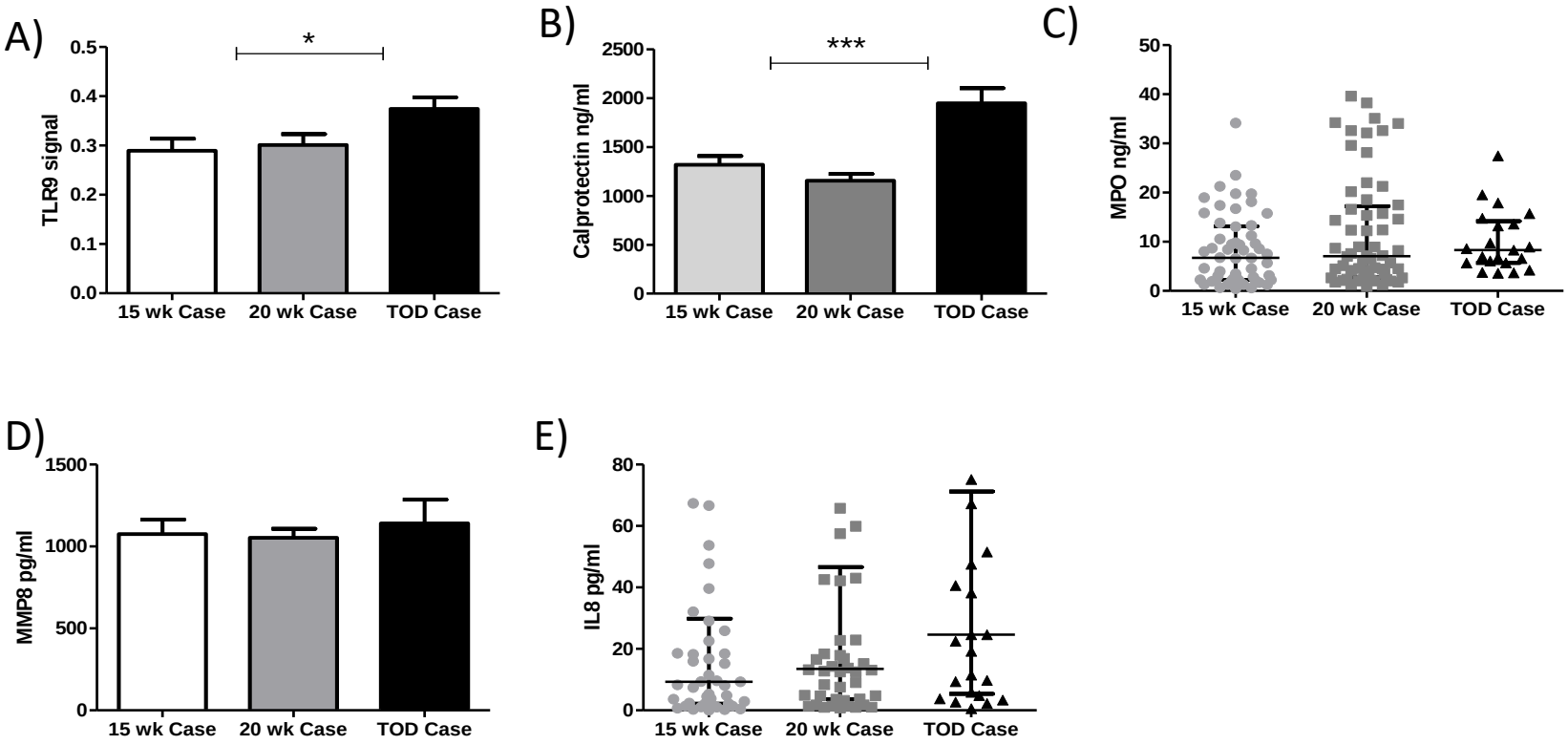


Figure 3.5: Neutrophil activation markers are increased across pregnancy in pre-eclampsia. TLR9 activity (a), Calprotectin (b), were both significantly increased across gestation in cases with pre-eclampsia (P=0.01). There was no significant increase in neutrophil activation markers MPO (c), MMP8 (d) and IL-8 (e) across gestation in cases with pre-eclampsia.

3.4.5 Determination of TLR9 activity and downstream markers of neutrophil activation in preterm cases versus term cases

We next explored if there was a difference in the neutrophil pro-inflammatory phenotype across all time-points in women who developed pre-eclampsia and delivered either before 37 weeks or after 37 weeks' gestation (Table 3.1). There was no significant difference in TLR9 activity in preterm pre-eclampsia compared to term pre-eclampsia across gestation. Similarly, there was no significance difference at each time-point when comparing expression of calprotectin, MPO and MMP8 in preterm pre-eclampsia versus term pre-eclampsia. Interestingly IL-8 expression is initially increased at 15 weeks' gestation in women who developed preterm pre-eclampsia, but the chemokine levels reduced as pregnancy progresses.

Table 3.1: Plasma levels of TLR9 activity, neutrophil activation markers and pro-inflammatory cytokine IL-8 in patients with preterm and term pre-eclampsia.

Neutrophil markers	15 weeks (Preterm N= 19)	15 weeks (Term N= 38)	P Value	20 weeks (Preterm N= 19)	20 weeks (Term N= 37)	P Value	TOD (Preterm N= 6)	TOD (Term N= 16)	P Value
TLR9 Signal	0.31 ± 0.05	0.27 ± 0.03	0.66	0.30 ± 0.06	0.29 ± 0.03	0.41	0.37 ± 0.03	0.39 ± 0.04	0.713
Calprotectin (ng/ml)	1043.15 ng/ml ± 126.19	1645.66 ng/ml ± 187.30	0.16	886.99 ± ng/ml 245.48	1167.96 ng/ml ± 77.80	0.57	1878.35 ng/ml ± 424.90	2055.57 ng/ml ± 170.62	0.77
MPO (ng/ml)	4.42 ng/ml [1.77-10.25]	6.58 ng/ml [2.11- 9.70]	0.56	4.55 ng/ml [2.48-8.74]	6.15 ng/ml [2.96- 21.46]	0.84	8.59 ng/ml [4.72-11.87]	8.33 ng/ml [5.67-14.78]	0.43
MMP8 (pg/ml)	1138.41 pg/ml ± 149.24	989.38 pg/ml ± 113.31	0.25	1097.48 pg/ml ± 108.58	1098.46pg/ml± 88.69	0.97	1775.73 pg/ml ± 466.30	1263.75 pg/ml ± 182.92	0.12
IL-8 (pg/ml)	32.09 pg/ml [7.40-192.91]	8.12 pg/ml [2.19-18.25]	0.17	22.92 pg/ml [4.76-188.69]	12.67 pg/ml [2.11-18.23]	0.32	24.63 pg/ml [2.68-47.59]	24.63 pg/ml [9.78-75.14]	0.81

Data is presented as mean ± SEM and median (25-75 percentiles) where applicable

3.5 Discussion

Pregnancy is associated with a maternal systemic inflammatory response; however, this response is exaggerated in pre-eclampsia. There has been a number of studies which have provided evidence of increased neutrophil activation in women with pre-eclampsia (312, 318, 319). In this present study, we investigated if circulating mediators (including mtDNA) (317), trigger TLR9 activity with the subsequent downstream activation of a neutrophil-mediated innate immune response in women with pre-eclampsia and healthy controls. We showed TLR9 activity is significantly increased in women with pre-eclampsia compared to healthy controls at time of disease. There was a corresponding increase in production of downstream neutrophil activation markers, calprotectin, myeloperoxidase, MMP8 and the pro-inflammatory cytokine IL-8 in women with pre-eclampsia compared to healthy controls at time of disease, indicating that complete activation of neutrophil pro-inflammatory phenotype only became evident late in pregnancy in pre-eclampsia.

The innate immune response plays a well described role in the pathophysiology of pre-eclampsia. Mitochondrial DAMPs such as mtDNA bind specific pattern recognition receptors such as TLR9 on the endosomal surface of the immune cells, in particular, neutrophils and activate an innate response. TLR9 receptors are also localised to trophoblasts and the villous stromal vascular endothelium and their expression is increased in pre-eclampsia (140). We have shown a significant increase in TLR9 activity using a reporter cell assay incubated with plasma taken at time of disease in pre-eclampsia cases compared to healthy controls. Furthermore, previous work in our lab examined mtDNA (as a marker of mitochondrial dysfunction) and reported that mtDNA was also significantly increased at time of disease in pre-eclampsia cases in the same study cohort (317). This indicates that the increase in

mitochondrial DAMP (mtDNA) at time of disease may activate a TLR9 mediated innate immune response in pre-eclampsia cases only. Exciting new work by He et al. has identified an additional role for TLR9 in pre-eclampsia where they established that TLR9 supressed angiogenesis in part by increasing sFlt-1 expression in a murine model of pre-eclampsia (320). Furthermore, research carried out in spontaneous hypertensive rats, established that circulating mtDNA may lead to the activation of the innate immune system through TLR9 (315). Research by Goulopoulou et al., has also shown preliminary data indicating that TLR9 activation provokes pre-eclampsia–like symptoms in pregnant rats (141) emphasising the pathogenic role of TLR9 in pre-eclampsia.

Calprotectin is a calcium binding protein that is located in the cytosol of neutrophils and is released upon neutrophil activation. In our study, calprotectin was significantly increased at time of disease compared to controls. This is in agreement with recent work by Akçum et al, who reported increased circulating calprotectin levels at term in women with pre-eclampsia, and interestingly found an additional increase in women with severe pre-eclampsia (319).

MPO is a lysosomal enzyme mainly produced and released by activated neutrophils. In this study, we showed a significant increase in MPO in cases as early as 20 weeks' gestation when compared with controls and this significant increase was equally evident at time of disease in pre-eclampsia. Previous studies measuring MPO in pre-eclampsia have reported conflicting results; some studies have reported no difference in MPO in pre-eclampsia in samples taken at 24 weeks or later in the third trimester (158, 321), whereas Gandley et al, reported a 3-fold increase in circulating MPO levels in women with pre-eclampsia compared to matched healthy controls at 32- 38 weeks' gestation (154). The variation in MPO levels in previous pre-

eclampsia studies may be due to small sample size. In our study we compared 60 cases and 120 matched controls, which to our knowledge is one of the largest studies measuring MPO in pre-eclampsia.

MMP8 is part of the MMP family which consists of 23 zinc and calcium dependent proteases that effect different mechanisms of the extracellular matrix. In recent years, MMPs have become a target of interest in pre-eclampsia due to their role in vascular function and remodelling (168, 322). Recent research shows strong evidence that MMP8 plays an important role in mediating endothelial cell angiogenesis (168). Furthermore, as endothelial dysfunction is a pathogenic characteristic of pre-eclampsia, dysregulated MMP8 production may play a crucial role in the disruption of angiogenesis in pre-eclampsia leading to endothelial dysfunction. In our study, there was an increase in circulating levels of MMP8 at TOD in women with pre-eclampsia cases compared to healthy controls.

Cytokines and chemokines have been extensively studied as markers of inflammation involved in the pathophysiology of pre-eclampsia. In this study, we showed a significant increase in circulating IL-8 levels at time of disease in cases compared to controls. IL-8 has previously shown to be increased in women with pre-eclampsia in the third trimester (323). Additionally, a recent study reported a significant increase in IL-8 in early gestation (5-15 weeks') in women who subsequently went on to develop pre-eclampsia. However, a limitation of their study was the small sample size (n=9) (179). Other studies investigating IL-8 throughout pregnancy reported similar results to ours with no significant increase evident in the second trimester (180, 181) but a significant increase in IL-8 is evident in the third trimester in women with pre-eclampsia compared to healthy controls.(182).

Preterm pre-eclampsia occurs before 37 weeks' gestation and is frequently regarded as a slightly different phenotype to pre-eclampsia occurring at term. Therefore, we investigated whether neutrophil activation triggers an altered innate immune response in preterm pre-eclampsia compared to term pre-eclampsia. In this study, there was no statistically significant difference in the concentration of neutrophil activation makers in preterm versus term pre-eclampsia respectively in our cohort.

When investigating the activation of a TLR9 mediated innate immune response across pregnancy, there was no significant difference in expression of any of the neutrophil markers measured in healthy controls. However, in pre-eclampsia, significant increases were seen in neutrophil activation markers, TLR9 and calprotectin, illustrating that the innate immune response may be initially triggered earlier in pre-eclampsia, but complete activation is not significantly evident until later in pregnancy. This is in agreement with recent studies of maternal inflammation (cytokines such as IL-6, TNF-α) in pre-eclampsia, whereby low level inflammation is evident early in pregnancies but is amplified in the third trimester of pregnancy (324-326). This current study shows that the TLR9 activation of the innate immune system may play a role in the pathophysiology of pre-eclampsia in late gestation.

3.6 Conclusion

Here we provide evidence that circulating plasma mediators may activate a TRL9-mediated innate immune response in pre-eclampsia. We show that the activity of TLR9, a receptor for mtDNA, is significantly increased at time of disease in pre-eclampsia. Subsequently we have shown increased production of neutrophil activation markers particularly late in pregnancy in pre-eclampsia. Finally, we have shown that possible activation of TLR9 by dysfunctional mitochondria may provoke an exaggerated neutrophil-mediated innate immune response in pre-eclampsia.

Chapter 4: Investigating the molecular mechanisms of the antioxidant L-ergothioneine in regulating mitochondrial function in an in vitro model of placental oxidative stress

Rachel D Williamson, Fergus P McCarthy, Robert N Kerley, Douglas B Kell, Louise C Kenny, Cathal M McCarthy. Molecular mechanisms of L-Ergothioneine as a mitochondrial targeted antioxidant for the treatment of pre-eclampsia. *Submitted* to *Placenta*

4.0 Chapter 4: Investigating the molecular mechanisms of the antioxidant L-ergothioneine in regulating mitochondrial function in an in vitro model of placental oxidative stress

4.1 Abstract

Introduction: Placental oxidative stress due to abnormal placentation is proposed to play a vital role in the pathophysiology of pre-eclampsia. To date, however, the results of testing antioxidant interventions in therapeutic trials have been overwhelmingly disappointing. There is preliminary evidence linking mitochondrial dysfunction as a potential mediator of exaggerated oxidative stress, evident in pre-eclampsia. The aim of this study was to investigate the potential therapeutic properties of L-ergothioneine, a promising and unusual antioxidant, in an *in vitro* model of oxidative stress.

Methods: Jeg-3 placental choriocarcinoma cells were pre-treated with 1mM L-ergothioneine, 20µM Mito-Tempo and 1mM N-acetylcysteine for 2 hrs and subsequently treated with 200µM H_2O_2 for 4 hrs. Cell viability, mitochondrial-specific superoxide production, mitochondrial mass and mitochondrial superoxide detoxifying enzymes were measured.

Results: Pre-treatment with L-ergothioneine significantly increased cytoprotection in Jeg-3 cells (91% ± 4.95% v 46.73 % ± 10.0%, n=3, P<0.01). L-ergothioneine significantly reduced mitochondrial-specific superoxide (100% v 48.36% ± 13.78 n=3; P≤0.01). Compared with MitoTempo and N-acetylcysteine, L-ergothioneine had a more pronounced effect across all measured parameters. PGC-1α (1.8-fold ± 0.3, n=3, P= 0.03) and UCP-1 (1.6 fold ± 0.3, n=3, P=0.03) were significantly

altered in cells pre-treated with L-ergothioneine and subsequently exposed to oxidative stress.

Discussion: Here we provide evidence of ERG's potential protective mechanisms in an *in vitro* model of oxidative stress. L-ergothioneine had more pronounced cytoprotective properties compared with either the mitochondrial-targeted antioxidant MitoTempo or N-acetylcysteine. L-ergothioneine alleviated the effects of the H_2O_2 oxidative insult on mitochondrial reactive oxygen species generation. Finally, we provide evidence that ERG may mediate its cytoprotective effects, at least in part, by regulating markers involved in mild mitochondrial uncoupling which dampen exaggerated reactive oxygen species production.

4.2 Introduction

Pre-eclampsia is characterised as de-novo hypertension in pregnancy (BP ≥140 mm Hg systolic or ≥90 mm Hg diastolic) at or after 20 weeks' gestation accompanied by proteinuria and/or evidence of maternal acute kidney injury (AKI), neurological features, haemolysis, liver dysfunction, or fetal growth restriction. It results in over 500,000 fetal and neonatal deaths and more than 70,000 maternal deaths globally (327). There is substantial evidence that defective placentation in early pregnancy is a vital event in the development of this condition (274). Furthermore, the ischemic placental microenvironment evident in pre-eclampsia is initially triggered by a reduction in placental perfusion due to oscillations in oxygen delivery to the placenta and fetus, which in turn results in exaggerated oxidative stress (266). Inflammation is also a significant factor (328). To date, research has shown that abnormally elevated oxidative stress is present in pre-eclampsia as early as 8-10 weeks' gestation (329).

There are many sources of reactive oxygen species (ROS) within the cell; however, mitochondria are the dominant cellular producer of ROS (330). There is growing evidence that mitochondrial dysfunction may play a role in the pathophysiology of pre-eclampsia (279, 317), where research has shown elevated levels of mtDNA in plasma from women with pre-eclampsia when compared with matched healthy controls (6). Mitochondrial DNA is generally sequestered inside mitochondria, however after physiological stressors such as trauma, infection, oxidative stress, mtDNA molecules are evident in circulation (136). Furthermore, there is evidence of diminished mitochondrial antioxidant defence in pre-eclampsia as early as 15 weeks' gestation (317, 331).

The placenta requires an extensive number of functional mitochondria to meet the elevated metabolic demands of pregnancy. To maintain the appropriate number, function and morphology, there needs to be a balance between fusion and fission (332, 333). Peroxisome proliferation-activated receptor ɣ co-activator 1α (PGC-1α) is a transcriptional coactivator that regulates numerous auxiliary biological functions associated with mitochondria, including antioxidant defence system, angiogenesis, cellular respiration and oxidative metabolism (334, 335) . Furthermore, PGC-1α directs a wide programme of mitochondrial gene expression of mitochondrial-ROS detoxifying enzymes including superoxide dismutase (SOD1, SOD2) and uncoupling protein-1 (UCP-1), which are directly induced by this transcriptional orchestrator (336). UCP-1 is a mitochondrial transporter present in the inner mitochondrial membrane of mitochondria with a well-known role in mediating adaptive thermogenesis (335) but more recently has been shown to regulate exaggerated mROS production in part by altering the proton gradient via mild uncoupling (337).

L-ergothioneine (ERG) is a dietary, water-soluble amino acid derived from histidine, cysteine and methionine. It is synthesised by various bacteria and fungi and taken up by higher organisms. Many physiological roles have been proposed for ERG including immune regulation, scavenging of free radicals and cation chelation (210). Furthermore, ERG has been shown to possess cytoprotective and antioxidant properties in both *in vitro* and *in vivo* models (208, 210, 211, 219, 221, 338-341), in addition to being involved in anti-inflammatory responses (342). ERG crosses the intestinal epithelium using the type 1 organic cation transporter SLC22A4 (previously known as OCTN1). This cation transporter's primarily role had been considered to be in the elimination of drugs from tissues such as the kidneys (215). SLC22A4 was initially considered to be a carnitine transporter, however it transports

ERG at a rate almost two orders of magnitude greater (216, 343), indicating that the ability to accumulate ERG was selected by evolution. ERG accumulates in conditions of high oxidative stress, indicating it may be an encouraging target for a therapeutic agent for diseases involving oxidative stress, such as pre-eclampsia (213, 344). Furthermore, no adverse effects were seen in pregnant Sprague Dawley rats administered ERG in their drinking water (231). ERG is a commercially available amino acid, with proven pharmacovigilance for consumption in humans (232) indicating its potential as a safe treatment for humans, as confirmed by the European Food Standards (237, 345) Agency.

The aim of this study is to investigate the mechanistic role of ERG in modulating mitochondrial function in an *in vitro* model of placental oxidative stress and assess its potential role as a novel therapeutic target for pre-eclampsia.

4.3 Materials and Methods

4.3.1 Cell culture and treatments.

The human JEG-3 choriocarcinoma placental cell line (ATTC), was grown in Ham's F-12 (Invitrogen) media supplemented with 10% Fetal bovine serum (FBS), 1% L-glutamine and 1% Penicillin Streptomycin (Biosciences). Cells were maintained at 37℃ and 5% CO_2 and were routinely passaged at 80% confluency. All experiments were performed on cells seeded 24-48 h in advance and serum starved for 1 hour prior to treatments. Cells were pre-treated initially with increasing concentrations of L-ergothioneine (1µM–1mM) and Mito-Tempo (1µM-100µM), and N-acetylcysteine (NAC) (1µM-1mM) 24hr. For co-treatments, cells were pre-treated with optimal concentrations of 1mM L-ergothioneine, 20 µM Mito-Tempo and 1µM N-acetylcysteine (NAC) for 2 hrs and 200µM H_2O_2 was then added for 4 hrs. The oxidative stressor insult (200µM H_2O_2) was chosen based on our previous *in vitro* investigations (109).

4.3.2 Assessment of Cell Viability using MTT assay

The thiazolyl blue tetrazolium bromide MTT assay assesses cell viability as it requires the metabolic conversion of MTT to an insoluble derivative formazan, thereby reflecting the number of viable cells present. Cells were treated as described in section 4.3.1 and were incubated at 37℃ with MTT solution for 4 hrs. Following treatment, 10 ul of MTT (final concentration 5 mg/ml) was added to each well and absorbance read at 570 nm, with 630 nm as a reference. Cell viability % = absorbance of each treated cells/absorbance of control- (DMSO-) treated cells x100.

4.3.3 Isolation of RNA and Real-time PCR analysis

RNA was extracted using the RNeasy mini-kit (Qiagen). Transcripts encoding Peroxisome proliferator-activated receptor-gamma coactivator (PGC-1α), superoxide dismutase (SOD1), SOD2, uncoupling protein-1 (UCP-1) and SLC22A4 were quantified by real-time PCR using StepONE Plus Detection system. Taqman assay (Applied Biosciences) and Sybr Green primers were used for quantification. The amounts of the target gene was normalised to the geometric mean of internal control gene 18S, and were determined using the comparative $2^{-\Delta\Delta CT}$ method (346).

4.3.4 Detection of mitochondrial superoxide by fluorescence microscopy

MitoSOX Red fluorescence reagent (Invitrogen) was used to measure intramitochondrial superoxide. This fluorogenic dye is specifically oxidised by superoxide anions in the mitochondria. This oxidation reaction produces a red fluorescent dye once bound to the mitochondrial DNA. Cells were treated as described in section 4.3.1. Media were then removed and 0.5μM MitoSOX red was added to the cells for 30 mins at 37˚C. Cells were then fixed and permeabilized prior to nuclear staining with DAPI. Mean fluorescence intensity was analysed using Image J software in at least 10 random fields of view and compared to DMSO controls.

4.3.5 Determination of mitochondrial mass

Mitochondrial mass was measured using MitoID green fluorescent marker (Enzo Life Sciences) according to the manufacturer's instructions. MitoID is a cell permeable small organic probe that is considered to localise to the mitochondria irrespective of any membrane potential they might possess. All experiments were performed at a cell

density of 50,000 cells per well in a 12 well plate. Cells were serum starved for 1 hour and subsequently pre-treated with either 1mM L-ergothioneine, 1mM NAC or 20 µM Mito-Tempo for 2 hrs. Thereafter, cells were treated with 200 µM H_2O_2 for 4 hrs.

4.3.6 Statistical analysis

Analyses were performed using GraphPad Prism. Data were presented as mean (±SEM), or fold change relative to control. Comparisons of data between *in vitro* groups were performed using t-test or analyses of variance (ANOVA) where appropriate to determine statistical significance between groups in *in vitro* studies unless otherwise specified. P values <0.05 were considered as statistically significant

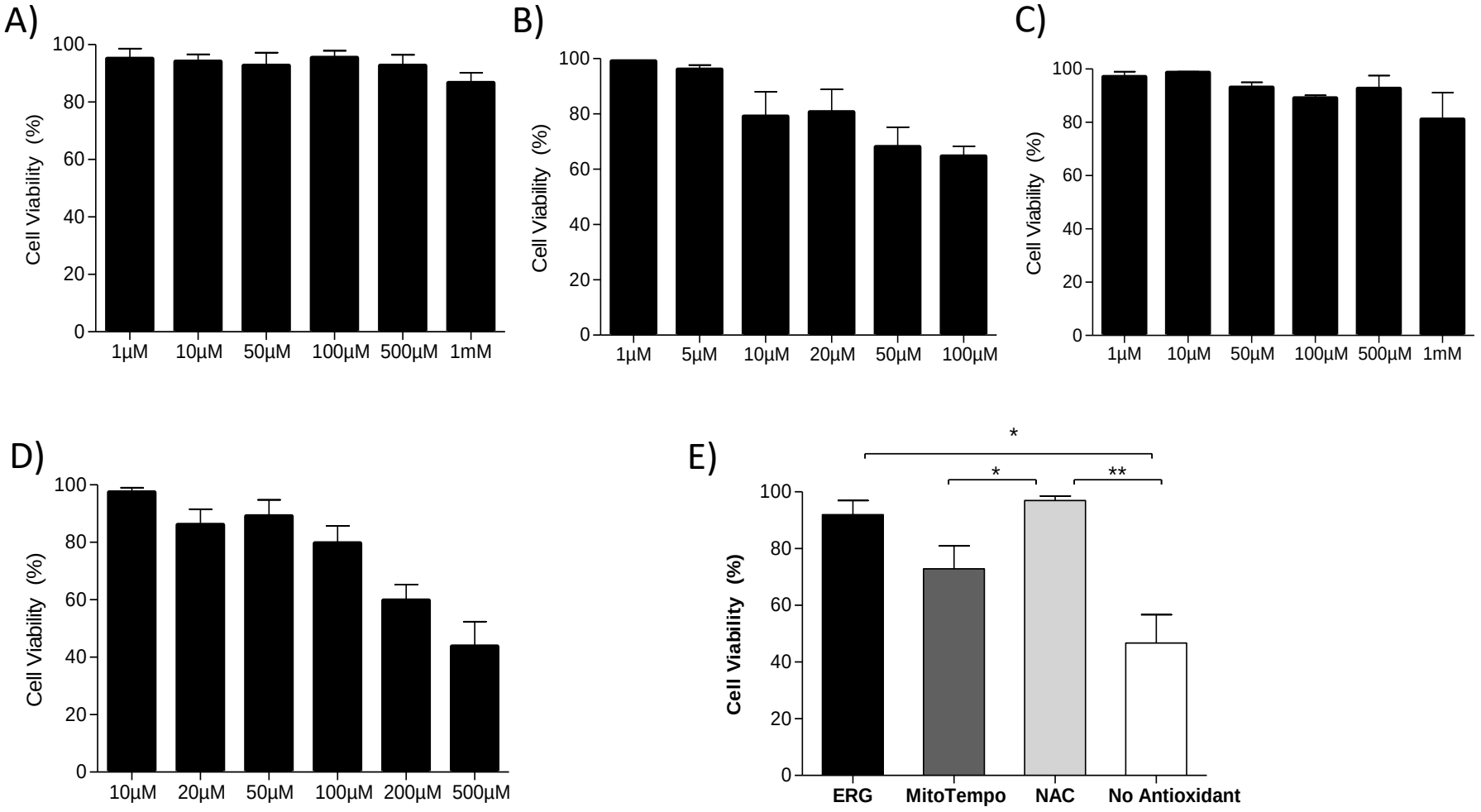
4.4 Results

4.4.1 Determine the preferred concentration of antioxidants on cell viability

To examine the preferred concentration of ERG, Jeg-3 cells were treated with a range of increasing concentrations (1μM to 1mM) for 24 hrs. In summary, 1mM was used for subsequent experiments (Figure 4.1A). Similar cell viability assays were performed with a mitochondrial-targeted antioxidant Mito-Tempo (1μM- 100μM) (Figure 4.1B) and NAC a non-mitochondrial antioxidant (1μM-1mM) (Figure 4.1C). In summary, 20μM Mito-Tempo and 1mM NAC were used in subsequent experiments.

4.4.2 Cytoprotective effects of ERG on H_2O_2 -induced cell death

The next set of experiments were designed to determine whether ERG had cytoprotective properties on H_2O_2-treated cells. H_2O_2 is commonly used as a cellular stressor representative of oxidative stress in *in vitro* models. 200 μM H_2O_2 was used for subsequent experiments (Figure 4.1D). Pre-treatment with 1mM ERG significantly rescued H_2O_2 –induced cell death (91% ± 4.95% v 46.73 % ± 10.0%, n=3, P= 0.01), when compared with the mitochondrial-targeted antioxidant Mito-Tempo (72% ± 8.1% v 46.73% ± 10%, n=3, P=0.11). Furthermore, ERG-mediated protective effect was similar to that of non-mitochondrial antioxidant NAC (96% ± 1.6% v 46.73% ± 10%, n=3, P= 0.007) (Figure 4.1E).


A)
Cell Viability (%)
100
80
60
40
20
0
1μM
10μM
50μM
100μM
500μM
1mM
B)
Cell Viability (%)
100
80
60
40
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0
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C)
Cell Viability (%)
100
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D)
Cell Viability (%)
100
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10μM
20μM
50μM
100μM
200μM
500μM
E)
*
*
**
Cell Viability (%)
100
80
60
40
20
0
ERG
MitoTempo
NAC
No Antioxidant

Figure 4.1: Cytoprotective effects of ERG, MitoTempo and NAC on H_2O_2 –induced-cell death. A-C) Dose dependent effect of ERG, MitoTempo and NAC on Jeg-3 cell viability was assessed using a MTT assay. Jeg-3 cells were treated with varying concentrations of ERG, MitoTempo and NAC for 24 hrs. Data are expressed as mean ± SEM. D) Dose dependent effect of H_2O_2 on Jeg-3 cell viability was assessed using a MTT assay. Jeg-3 cells were treated with varying concentrations of H_2O_2 for 24 hrs. Data are expressed as mean ± SEM. E) ERG is protective of H_2O_2-induced cell death in Jeg-3 cells. Cells were pre-treated with ERG, MitoTempo or NAC for 2 hrs and subsequently exposed to 200µM H_2O_2 for 4 hrs, cell viability was assessed by MTT assay. Data are expressed as mean ±SEM. (*P<0.05, **P<0.01; v Control). Data are representatives of 3 independent experiments.

4.4.3 ERG reduces mitochondrial specific ROS in H_2O_2 treated cells

Increased production of mitochondrial-specific ROS (mROS) is inherently linked to mitochondrial dysfunction. To examine mitochondrial-specific superoxide production, cells were labelled with MitoSOX Red fluorescent dye and quantified by fluorescent microscopy. Mitochondrial-specific superoxide was significantly reduced in cells pre-treated with ERG (100% v 48% ± 14%, n=3; $P<0.01$) compared with control cells (Figure 4.2). Similarly, MitoTempo significantly reduced mitochondrial-specific superoxide production, (100% v 51% ± 12%, n=3; $P= 0.01$), while NAC didn't significantly reduce mROS production respectively (100% v 68% ± 7%, n=3 $P= 0.08$).

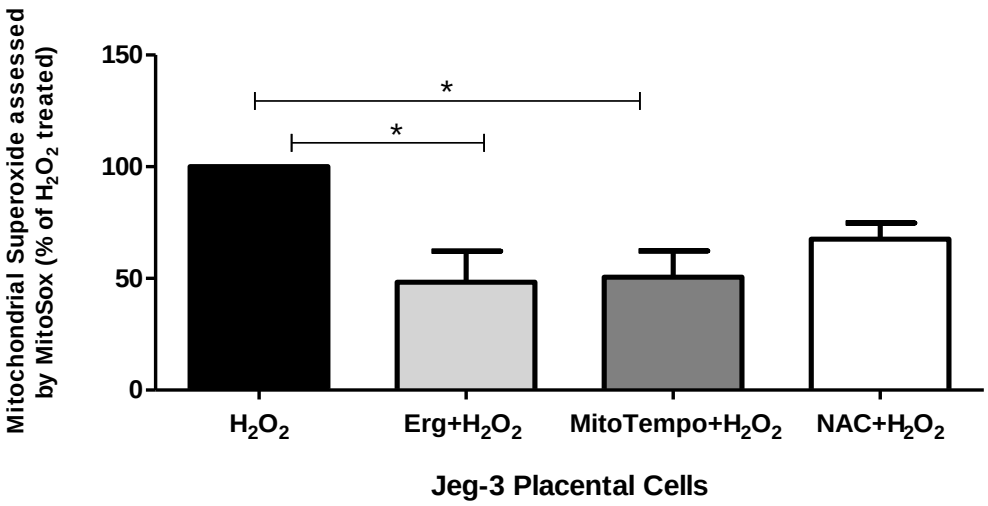

Figure 4.2: ERG reduced mitochondrial superoxide production in Jeg-3 cells. Jeg-3 cells were pre-treated with ERG, MitoTempo or NAC for 2 hrs and subsequently treated with H_2O_2 for 4hrs and mitochondrial-specific superoxide was detected using fluorogenic MitoSox Red dye. MitoSox Red generation was quantified using Image J software. Data are the mean of 10 independent experiments and are expressed as difference in percentage pixel intensity between study groups ± SEM. *P<0.01.

4.4.4 Effect of ERG on mitochondrial mass in Jeg-3 treated cells

The effect of ERG on mitochondrial mass in JEG-3 cells was measured using fluorescent MitoID Green. MitoID Green accumulates in the mitochondria and becomes fluorescent in a lipid environment, fluorescence was quantified by microscopy. There was no significant change in mitochondrial mass in cells pre-treated with ERG compared to control cells (85% ± 7% v 100%, n=3, P=0.06). Equally, MitoTempo (84% ± 27% v 100%, n=3, P= 0.35) or NAC treated cells didn't significantly alter mitochondrial mass when compared to control cells (81% ± 7% v 100%, n=3; P=0.09). (Figure 4.3).

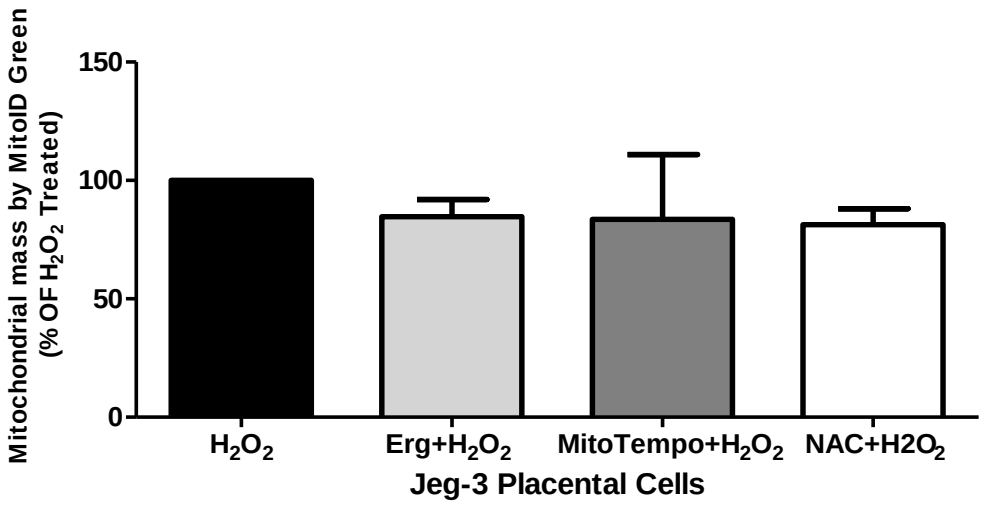

Figure 4.3: Effect of antioxidants on mitochondrial mass in H_2O_2 treated cells.

Jeg-3 cells were pre-treated with ERG, MitoTempo or NAC for 2 hrs and subsequently treated with H_2O_2 for 4hrs. Mitochondrial mass was determined using fluorogenic MitoID green dye. Mitochondrial mass was quantified using Image J software. Data are the mean of 3 independent experiments and are expressed as difference in percentage pixel intensity between group's ± SEM. *P <0.05.

4.4.5 ERG regulates endogenous UCP-1 expression in placental cells

We assessed the effect of ERG treatment on expression of PGC-1α and its directly regulated mitochondrial detoxifying enzymes SOD1, SOD2 and UCP-1. We also measured SLC22A4 expression in ERG-treated cells. This study showed that ERG treatment did not have a significant effect on the expression of PGC-1α, SOD1, SOD2, SLC22A4 in Jeg-3 cells compared with untreated cells. (Figure 4.4). However, UCP-1 expression was increased in cells treated with ERG (2 fold ± 0.5, n=3, P=0.04) compared to untreated cells.

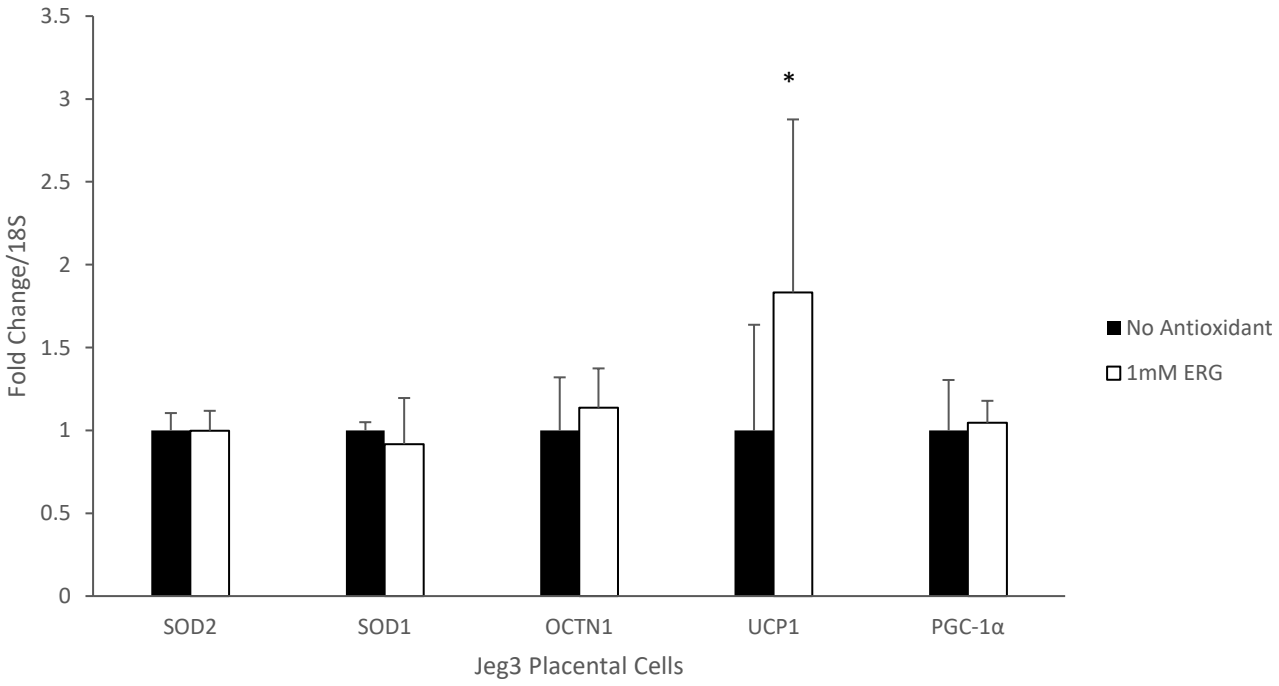

Figure 4.4: Effects of antioxidants on endogenous expression of mitochondrial-detoxifying enzymes in untreated Jeg-3 cells. Jeg-3 cells were treated with antioxidants as described for 24hrs and placental expression of markers of mitochondrial-detoxifying enzymes were quantified by real-time PCR. Gene expression of PGC-1α, UCP-1 SOD1, SOD2, and SLC22A4 were determined in Jeg-

3 cells. Data are representative of 3 independent experiments. Data are mean fold change compared to untreated Jeg-3 cells ± SEM. *P<0.01.

4.4.6 ERG alters the profile of mitochondrial superoxide detoxifying enzymes in response to oxidative stress

ERG significantly increased the expression of PGC-1α (1.8-fold ± 0.3, n=3, P= 0.03) compared to control cells. (Figure 4.5). Additionally, Mito-Tempo (1.5 fold ± 0.2, n=3, P=0.04) and NAC (1.4 fold ± 0.1, n=3, P=0.002) significantly increased PCG-1α expression, albeit with a reduced effect. UCP-1 is known to mediate mROS production in part by mild uncoupling therefore, we wanted to investigate the effect of ERG treatment on UCP-1 expression. ERG significantly increased the expression of UCP-1 (1.6 fold ± 0.3, n=3, P=0.03) compared with controls (Figure 4.5). Mito-Tempo (0.7 fold ± 0.33, n=3, P= 0.66) and NAC (0.76 fold ± 0.38, n=3, P= 0.61) did not alter UCP-1 expression. The expression of SOD1, SOD2 or SLC22A4 did not change in response to any pre-treatment strategy.

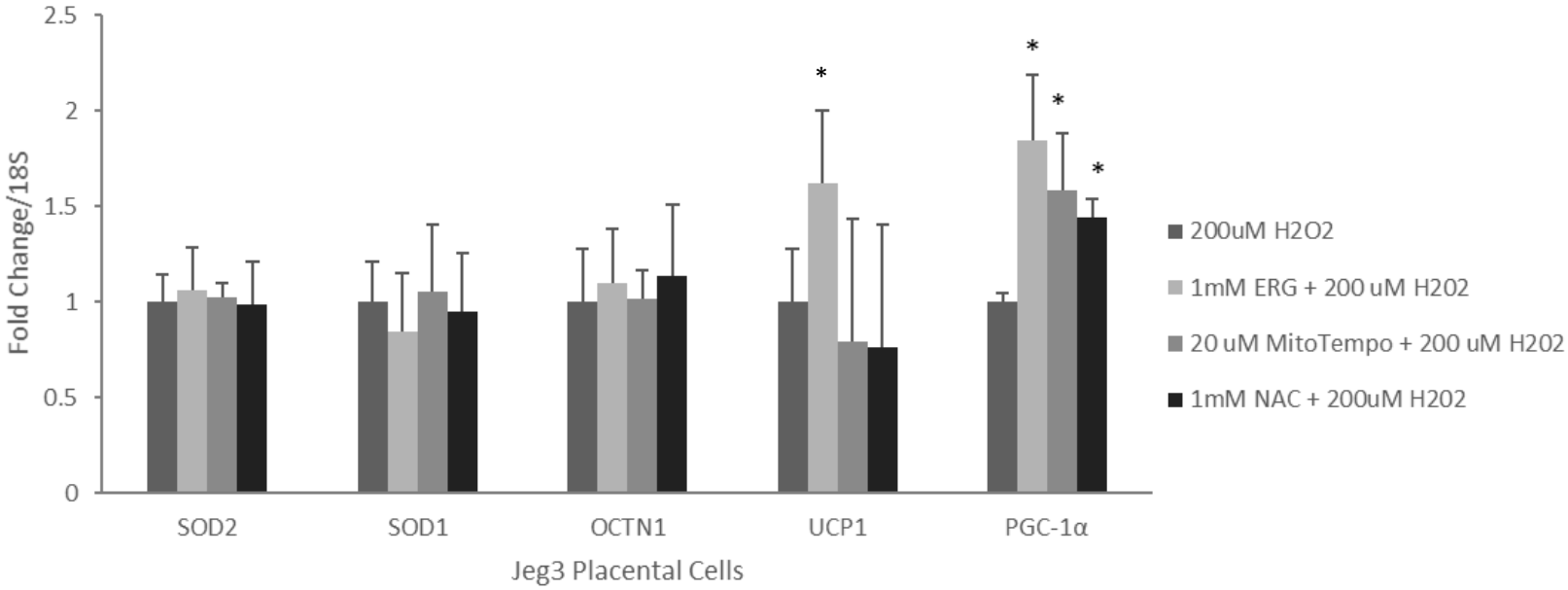

Figure 4.5: Effects of ERG, MitoTempo and NAC on placental expression of mitochondrial-detoxifying enzymes in H_2O_2 treated cells: Jeg-3 cells were pre-treated with ERG, MitoTempo and NAC for 2 hrs prior to exposure to H_2O_2 for 4 hrs and gene expression of markers of mitochondrial-detoxifying enzymes were quantified by real-time PCR. The amounts of amplified products were expressed relative to geometric mean of 18S internal controls. Data are mean fold change compared to controls ± SEM. *P<0.01. Data are representative of 3 independent experiments.

4.5 Discussion

To date, research has demonstrated that oxidative stress is a prime mediator in the pathophysiology of pre-eclampsia, and more recently there has been a specific focus on the role of mitochondrial dysfunction in regulating oxidative stress in pre-eclampsia (109, 204). However, a number of vitamin antioxidants have been overwhelmingly unsuccessful as treatment strategies for pre-eclampsia (187, 347). This may be due to the fact that they remain sequestered in the cytoplasm and fail to reach the mitochondria, therefore restricting their capacity to alter the oxidative damage seen in pre-eclampsia, or because in the presence of free iron they are actually pro-oxidant (330). The overall aim of this study was to explore the mechanism of action of a novel, potential mitochondrial antioxidant, L-ergothioneine in regulating mitochondrial function in an *in vitro* model of placental oxidative stress and subsequently elucidate its role as a potential therapeutic target for pre-eclampsia.

Initially, we wanted to investigate the cytoprotective effects of ERG on H_2O_2-induced oxidative damage in Jeg-3 cells. We observed that 1mM ERG significantly preserved cell viability in response to the oxidative stress. Similarly, Paul et al, showed ERG exerted cytoprotective effects on HeLa cells following H_2O_2 treatment. Furthermore, these authors indicated that depletion of SLC22A4 markedly augmented sensitivity to pyrogallol-induced cell damage and death, reinforcing the physiologic antioxidant role of ERG (209). ERG has previously been used for the treatment of oxidative stress in human brain microvascular endothelial cells (HBMECs) where Li et al, showed ERG reduced cell death as well as oxidative stress (229). MitoTempo is an extensively studied mitochondrial-targeted antioxidant (109); therefore, we wanted to compare the efficacy of ERG in protecting cell

viability in comparison to the established MitoTempo. We demonstrated that ERG had increased cytoprotective properties when compared with MitoTempo.

In the present study, we showed that ERG significantly reduced mitochondrial-specific ROS production in Jeg-3 cells. Similarly, MitoTempo significantly reduced mROS production but with less potency than ERG. We have previously shown that MitoTempo also reduced mROS production in HUVEC cells incubated with plasma from women with pre-eclampsia (109). In comparison, in this *in vitro* model, ERG appeared to be more effective than was the non-mitochondrial antioxidant NAC, in reducing mROS generation, highlighting that ERG may combat placental oxidative stress evident in pre-eclampsia, by regulating mitochondrial reactive oxygen species production.

Mitochondrial biogenesis is involved in the control of cell metabolism, signal transduction and regulation of mROS production. Mitochondrial mass describes the equilibrium between rates of biogenesis and degradation (348). We measured mitochondrial mass and found no significant difference in Jeg-3 cells pre-treated with ERG which would suggest that the dampening of mROS upon ERG treatment is not directly related to a disruption in mitochondrial dynamics. We previously reported that mitochondrial-targeted antioxidant MitoTempo reduced mROS generation without altering mitochondrial mass in pre-eclampsia plasma treated (109), a similar effect was also evident in placental cells upon MitoTempo treatment.

PGC-1α is co-induced with numerous ROS-detoxifying enzymes upon exposure of cells to an oxidative insult and is often required for the activation of many these ROS-detoxifying enzymes, including SOD1, SOD2 and UCP-1 (336). We subsequently investigated if ERG altered the expression profile of mitochondrial-

ROS detoxifying enzymes in response to H_2O_2-induced oxidative stress. We identified a significant increase in expression of both PGC-1α and its downstream target UCP-1, in cells pre-treated with ERG. PGC-1α has previously been shown to be significantly reduced in placental tissue in women with pre-eclampsia (349), additionally, there was significant evidence of dysregulation of mitochondrial biogenesis in these placental tissues. UCP-1 is directly regulated by PGC-1α (336) and has recently been shown to regulate mROS production, in addition to its recognised role in adaptive thermogenesis (350, 351). Mild uncoupling due to increased UCP-1 expression may participate in an antioxidant defence mechanism to dampen exaggerated mROS production. Proton leakage back to the mitochondrial matrix from the intermembrane space induced by UCP-1, alters membrane potential and decreases mROS production (Figure 4.6). (351).

UCP-1 expression was also significantly increased by ERG treatment without exposure to H_2O_2-induced oxidative stress, suggesting that ERG treatment may prime trophoblast cells to respond more effectively to oxidative insult. While both MitoTempo and NAC had a marginal effect on PGC-1α expression, there was no additional effect on UCP-1 expression suggesting that ERG treatment may recruit alternative co-repressors or co-activators which amplify the UCP-1 response.

Under non-pathological circumstances, the production of mROS is strictly regulated by many antioxidant systems in order to sustain redox-signaling homeostasis. We subsequently investigated the effect of ERG on both endogenous and H_2O_2 - induced expression of SOD1 and SOD2 antioxidants in JEG-3 cells and found no significant effect of ERG in either physiological state. Similarly, MitoTempo or NAC had no significant effect on the expression of either antioxidant. We have previously shown that MitoTempo-mediated reduction of mROS in HUVEC cells also didn't directly alter the expression of SOD antioxidants.

Here we provide evidence that the antioxidant ERG has significant cytoprotective properties in response to oxidative stress. ERG significantly reduced the generation of mROS without altering mitochondrial mass or antioxidant expression. This study presents novel data that ERG may be mediating its effects via increasing UCP-1 expression which induces mild coupling and subsequent dampening of inflated mROS production. Finally, our study provides evidence that directly targeting mitochondrial superoxide scavenging with ERG could be a therapeutic candidate for the treatment of pre-eclampsia.

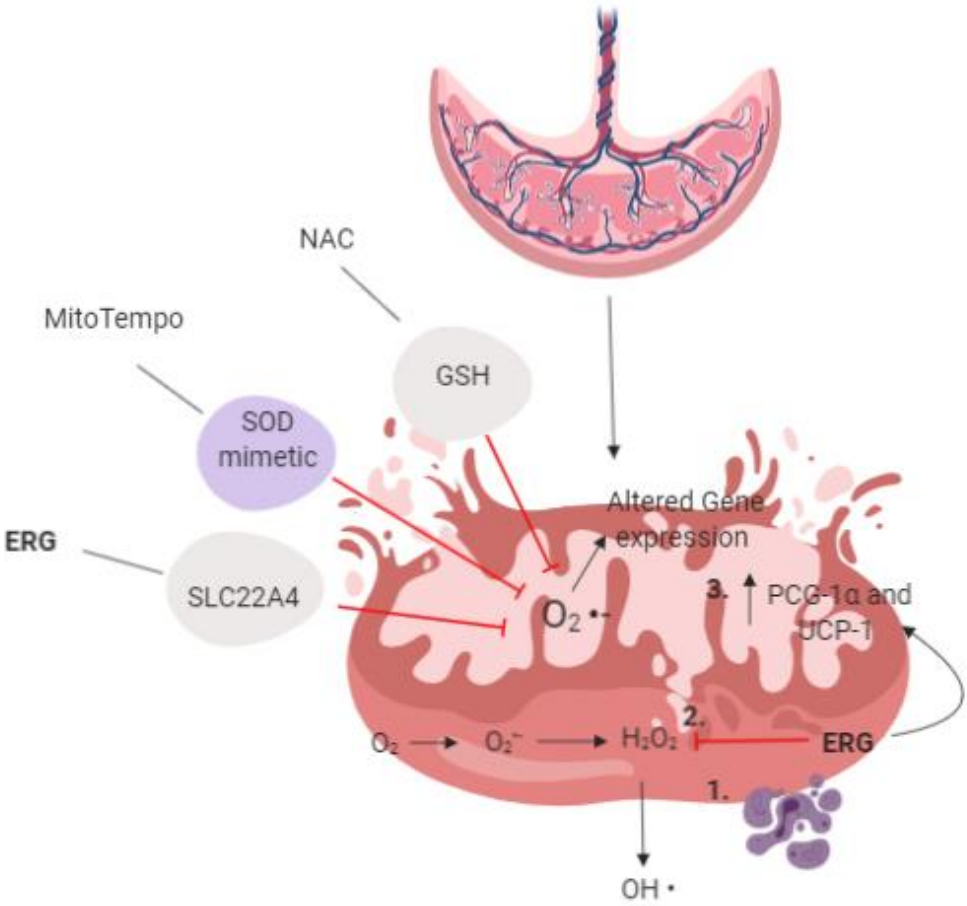

Figure 4.6: Proposed mechanism of action of ERG in the placenta. There is evidence that links the potential role of ERG as: 1. A cytoprotective compound against H_2O_2 induced cell death. ERG works through SLC22A4 transporter. 2. ERG directly scavenges mitochondrial-specific reactive oxygen species. 3. ERG modules mild uncoupling via alteration in expression of mitochondrial markers PGC-1α and UCP-1. This figure was generated using Biorender (https://app.biorender.com).

Chapter 5: L-ergothioneine as a potential therapeutic intervention in the treatment of Pre-eclampsia.

Rachel D Williamson, Fergus P McCarthy, Douglas B Kell, Louise C Kenny, Cathal M McCarthy. L-ergothioneine as a potential therapeutic intervention in the treatment of Pre-eclampsia. *Accepted pending minor changes* to *Hypertension*

5.0 Chapter 5: L-ergothioneine as a potential therapeutic intervention in the treatment of Pre-eclampsia.

5.1 Abstract

Introduction: Pre-eclampsia is a multifactorial disorder of pregnancy and abnormal placentation and the resultant placental ischemia microenvironment is thought to play a crucial role in its pathophysiology. Placental ischemia due to fluctuations in the delivery of oxygen results in oxidative stress, and recent evidence suggests that mitochondrial dysfunction may be a prime mediator of oxidative stress in response to placental ischaemia. However, large clinical trials of therapeutic antioxidants for the treatment of pre-eclampsia have been disappointing. L-ergothioneine, an unusual amino acid derived from histidine, has important cytoprotective and antioxidant properties under conditions of high oxidative stress.

Objective: In this study, we investigated the potential therapeutic effects of administration of L-ergothioneine in the reduced uterine perfusion pressure RUPP rat model of pre-eclampsia.

Method: L-ergothioneine (25mg/kg/day) was administered to rats on gestational day 11. On gestational day 14 RUPP surgery was performed and on gestational day 19, blood pressure (mean arterial pressure), fetal growth were measured. Kidney tissue samples were analysed for production mitochondrial-specific H_2O_2 *in vivo*.

Results: L-ergothioneine ameliorated hypertension and significantly increased pup weight in RUPP rats. Mitochondrial-specific H_2O_2 was significantly increased in kidney tissue in RUPP rats compared with sham group. L-ergothioneine treatment significantly reduced mitochondrial-specific H_2O_2 generation in kidney tissue from

RUPP rats. L-ergothioneine significantly decreased circulating levels of anti-angiogenic sFlt-1 in RUPP rats.

Conclusion: This novel data supports the potential for a mitochondrial targeted antioxidant approach for the treatment of pre-eclampsia.

5.2 Introduction

Pre-eclampsia is a multisystemic disorder of pregnancy which results in high blood pressure accompanied by and/or proteinuria, acute kidney injury (AKI), liver dysfunction fetal growth restriction (FGR) after 20 weeks' gestation (327), which affects more than eight million pregnancies worldwide annually and is the leading cause of maternal death (273). Despite extensive research, the exact pathophysiological mechanisms underlying this syndrome remain poorly elucidated. Nonetheless, defective placentation is strongly considered to be a critical event in the pathology of the disorder. Failure to remodel spiral arteries results in high-pressure blood-flow mediated placental damage and intermittent fluctuations in oxygen delivery, which expose the placenta to oxidative stress (266). The resultant placental ischaemic microenvironment is inherently linked to increased production and secretion of deleterious soluble mediators that provoke extensive maternal inflammation and endothelial dysfunction (352). Several reports have observed higher levels of markers of oxidative stress (F_2-isoprostanes, nitrotyrosine and 4-hydroxynonenal staining) in placental tissue from pre-eclamptic pregnancies compared with uncomplicated pregnancies (353-356).

While there are a number of different cellular sources of reactive oxygen species (ROS), mitochondria are the major cellular producers of ROS. Furthermore, in terms of pre- eclampsia, there is growing evidence incriminating mitochondrial dysfunction in its underlying pathophysiology (279). Initial studies showed increased mitochondrial lipid peroxidation and enhanced susceptibility to oxidative damage in placental tissue of pregnancies complicated by pre- eclampsia (104). More recently, work has confirmed this association with strong evidence of perturbation of

mitochondrial function in the metabolite profile of plasma samples taken at 15 week's gestation from patients who subsequently developed pre-eclampsia (66).

There is significant evidence for the pathogenic role of oxidative stress in the development of pre-eclampsia, yet clinical trials of antioxidant interventions were disappointing and not clinically effective in treating the disorder. One very plausible explanation is that these antioxidants missed the intracellular location of ROS production, namely the mitochondria; hence they have failed to alleviate the pathological oxidative damage. L-ergothioneine (ERG) is an unusual thio-histidine betaine amino acid and is a naturally occurring anti-oxidant discovered over a century ago in the rye ergot (208-210, 357). The predominant role of ERG, via a variety of mechanisms is to serve as an antioxidant and cellular protectant against various kinds of reactive oxygen species (358). Additionally, there has been some circumstantial evidence that ERG localises to mitochondria (216) and may dampen exaggerated mitochondrial-specific ROS in response to oxidative stress.

Therefore, we aimed to investigate the role of ERG as a potential therapeutic target for pre-eclampsia using the reduced uterine perfusion pressure (RUPP) model in pregnant rats. The placental ischaemic RUPP model has numerous features of pre-eclampsia that are evident in women and has been used as a preclinical model for the investigation of novel therapeutic targets for the treatment of pre-eclampsia (240, 359). Moreover, we additionally wanted to examine if ERG ameliorated the clinical characteristics of pre-eclampsia in part via regulation of mitochondrial-specific ROS production. Therefore, using a novel ratiometric mass spectrometry probe MitoB that specifically accumulates in mitochondria and generates a MitoP phenol product upon reaction with H_2O_2 which can be subsequently be analysed *ex vivo* by mass

spectrometry, we sought to measure levels of mitochondrial-specific H_2O_2 in the kidney *in vivo.*

5.3 Materials and Methods

5.3.1 Animals

Sprague Dawley-timed pregnant rats were supplied and maintained by the University College Cork Biological Services Unit. Animals were maintained at a temperature of 21˚C, with a 12-hour light/dark cycle and free access to food and tap water. All the procedures were performed in accordance with National Guidelines and the European Directive 2010/63/EU, under an authorization issued by the Health Products Regulatory Authority Ireland and approved by the Animal Ethics Committee of University College Cork (AE19130/P037).

5.3.2 RUPP procedure

The RUPP procedure is a well-established surgical model for studying the link between placental ischemia and hypertension in the pregnant rat and has been previously described in detail (240, 253). In brief, on gestational day (GD) 14, RUPP reduction in blood flow to the uteroplacental unit was achieved by placing silver clip (0.2 mm ID) on the abdominal aorta (1 clip) above the iliac bifurcation and furthermore, 2 clips (0.1 mm ID) were carefully placed around the left and right ovarian arteries. Sham surgery was performed as controls, which involved abdominal incision but didn’t involve insertion of any clips on either abdominal aorta or ovarian arteries. On GD18, a chronic indwelling catheter was inserted into the carotid artery and on GD19 mean arterial blood pressure (MABP) was recorded in conscious animals.

5.3.3 L-ergothioneine *In vivo* experimental protocol

Four experimental groups were used to investigate the effect of administration of ERG in the RUPP rat model of pre-eclampisa. Pregnant rats were divided into Sham (n=8), Sham+ERG (n=8) or RUPP (n=8), and RUPP+ERG (n=8). ERG was administered at (25mg/kg/day) in their drinking water on GD11 until the end of the experiment on GD19. The dose for ERG (25mg/kg/day) were selected based on previously published rodent studies using this antioxidant (360, 361). L-ergothioneine were provided by Tetrahedron (Paris, France; www.tetrahedron.fr).

5.3.4 Plasma Collection

Blood collected from EDTA vacutainers were centrifuged at 2000g and 2400g for 10 minutes respectively at 4˚C, plasma was removed and stored at -80˚C for further analysis.

5.3.5 Urine analysis

On GD18, each rat was singularly housed in a metabolic cage and urine collected overnight. All samples were stored immediately following collection at -80ºC. Microalbumin creatinine ratios were calculated following measurement of albumin using an immuno-turbidimetric test for the quantitative determination of albumin in an OLYMPUS AU5832 analyser® and creatinine using a kinetic colour test (Jaffé method) (362) for the quantitative determination of creatinine in urine. Similarly, protein creatinine ratios were calculated following measurement of protein by adding benzethonium chloride which resulted in the formation of a fine suspension, which was then quantified turbidimetrically at 525 nm using an OLYMPUS analysers®.

5.3.6 Measurement of anti-angiogenic protein sFlt-1 by ELISA

Circulating sFlt-1 concentration in plasma samples from all experimental groups were quantified by enzyme-linked immunosorbent assay (ELISA) using a Quantikine sFlt-1 immunoassay (R&D Systems) as per manufacturers' instructions.

5.3.7 Isolation of RNA and Real-time PCR analysis

RNA was extracted from placental tissue using the Trizol method. SLC22A4 (ERG transporter), superoxide dismutase 1 (SOD1), SOD2, and uncoupling protein-1 (UCP-1), PCG-1α and Nrf2 gene expression was quantified by Real-time PCR using StepONE Plus Detection system. Taqman assays (Applied Biosciences) and Sybr Green primers were used for quantification. The amounts of target gene normalised to geometric mean control of 18S were determined using $2^{-\Delta\Delta CT}$.

5.3.8 Isometic Myography

In all groups, third order mesenteric arteries were dissected and mounted on a 4-channel wire myograph (Model 610 mol/L Danish Myo Technology) containing oxygenated (95% O_2 and 5% CO_2) physiological salt solution (PSS) at 37˚C. Vessels were normalized to achieve a transmural pressure of 100mmHg using the DMT Normalization software. Isometric tension was recorded and displayed using Powerlab Chart Software (AD Instruments). The viability of the smooth muscle was examined by the addition of a 123 mmol/L KCl solution. After PSS washes, concentration responses were carried out with thromboxane mimetic U46619 (9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$; 10^{-9} to 10^{-5} mol/L) ,and either Bradykinin (BK; 10^{-8} to 10^{-5} mol/L), Acetylcholine (ACh; 10^{-8} to 10^{-5} mol/L), or Sodium Nitroprusside (SNP; 10^{-8} to 10^{-5} mol/L) respectively (259, 363).

5.3.9 Measurement of mitochondrial H_2O_2 in the RUPP model *In vivo*

Mitochondrial hydrogen peroxide was measured *in vivo* by Cayman Chemical using Cayman's Hydrogen Peroxide Ratiometric MaxSpec kit based on the MitoB mass spectrometric probe as described previously (364). Briefly, 75 nmol MitoB in 50µl saline was administered by tail-vein injection to rats in the 4 experimental groups on GD19, 4 hours prior to end of the experiment. At the end of the procedure, kidney tissues were dissected out, snap frozen and stored at −80 °C. For mitochondrial H_2O_2 analysis, kidney tissues were homogenised, spiked with deuterated internal standards and MitoB and its product MitoP were extracted using acetonitrile/formic acid. MitoB and MitoP present in kidney tissue were measured using UPLC Xevo TQD mass spectrometer (Waters) and the amounts of MitoP and MitoB in each sample was determined relative to a standard curve. The MitoP/MitoB ratios for each sample were then calculated.

5.3.10 Statistical analysis

All data are expressed as mean ± SEM, or fold change relative to control. Analysis was performed using GraphPad Prism. Data was represented as mean (±SEM) and comparisons of data between cases and controls were performed using an unpaired t-test. P values <0.05 were considered as statistically significant.

5.4 Results

5.4.1 L-ergothioneine ameliorated RUPP induced hypertension

MABP was significantly increased in the RUPP group compared with sham group (130.87 ± 3.54 mmHg vs 119.99 ± 6.27 mmHg, P = 0.02; Figure 5.1). Furthermore, administration of ERG significantly reduced MABP in the RUPP rats (130.87 ± 3.54 mmHg v 118.38 ± 4.86 mmHg) (P= 0.05; Figure 5.1). MABP in sham rats treated with ERG was not different from RUPPs treated with ERG, indicating the beneficial effect of ERG antioxidant in reducing blood pressure in response to placental ischemia. There was no significant difference in microalbumin:creatinine (MCR) ratio in the RUPP group compared to Sham group, equally, there was no significant difference in the MCR ratio in the RUPPs treated with ERG (Figure 5.2).

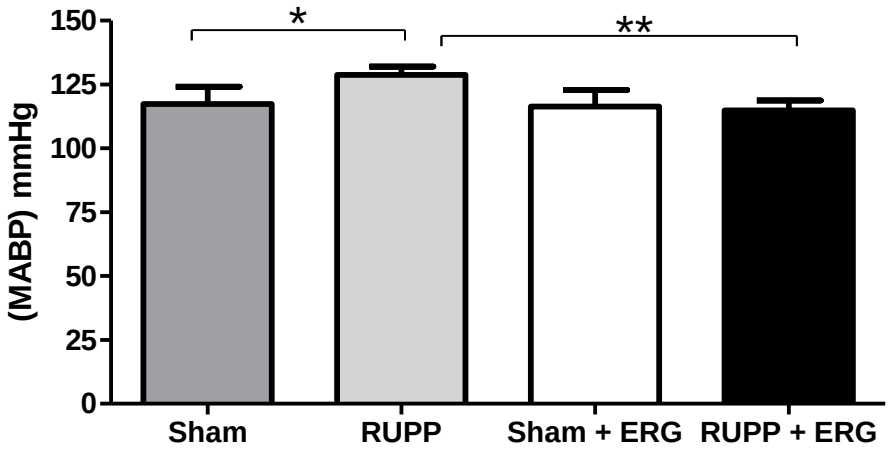

Figure 5.1: L-ergothioneine attenuates blood pressure in reduced uterine perfusion pressure (RUPP) rats: a) RUPP rats (n=8) showed significantly elevated mean arterial blood pressure (MABP) compared with sham group (n=8). L-ergothioneine (25mg/kg/day) reduced MABP in RUPP rats (n=8). Data is presented as mean ± SEM, P<0.05

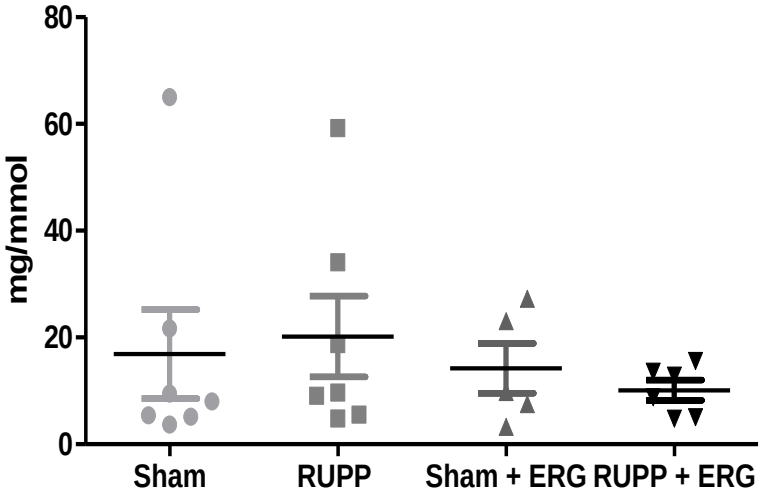

Figure 5.2: L-ergothioneine did not alter microalbumin:creatinine ratio in RUPP rats: Administration of L-ergothioneine (25mg/kg/day) did not significantly affect microalbumin:creatinine ratio in RUPP rats. Data is presented as mean ± SEM, $P<0.05$.

5.4.2 Effect of L-ergothioneine of fetal weight

Fetal weight was significantly decreased in the RUPP group compared to the Sham group (1.80 ± 0.04 g v 1.99 ± 0.03 g; P= 0.0004; Figure 5.3A). Administration of ERG significantly rescued fetal growth restriction in the RUPP rats (1.98 ± 0.03 g v 1.80 ± 0.04g, P= 0.0006; Figure 5.3A). Placental weights were significantly reduced in RUPP group compared to Sham group (0.43 ± 0.01 g v 0.50 ± 0.01 g; P= 0.002, Figure 3B). The administration of ERG had no effect on placental weight in the RUPP rats (0.42 ± 0.007 g v 0.43 ± 0.01 g) (Figure 5.3B). A significant decrease in pup number was observed in the RUPP group compared to Sham group (10.5 ± 1.40 v 13.5 ± 1.08; P= 0.02, Figure 5.3C). There was no significant difference in pup number in RUPPs treated with ERG (12.87 ± 1.60 v 10.5 ± 1.40). Pup weight was not significantly different between Sham or RUPPs treated with ERG, indicating the beneficial effect of mitochondrial antioxidants in rescuing fetal weight during placental ischemia. There was no significant difference in the crown to rump length (CRL) of pups in any of the studied groups (Figure 7.S1. Supplementary). Finally, maternal weight did not differ between any of the studied groups. (Figure 6.S1.Supplementary).

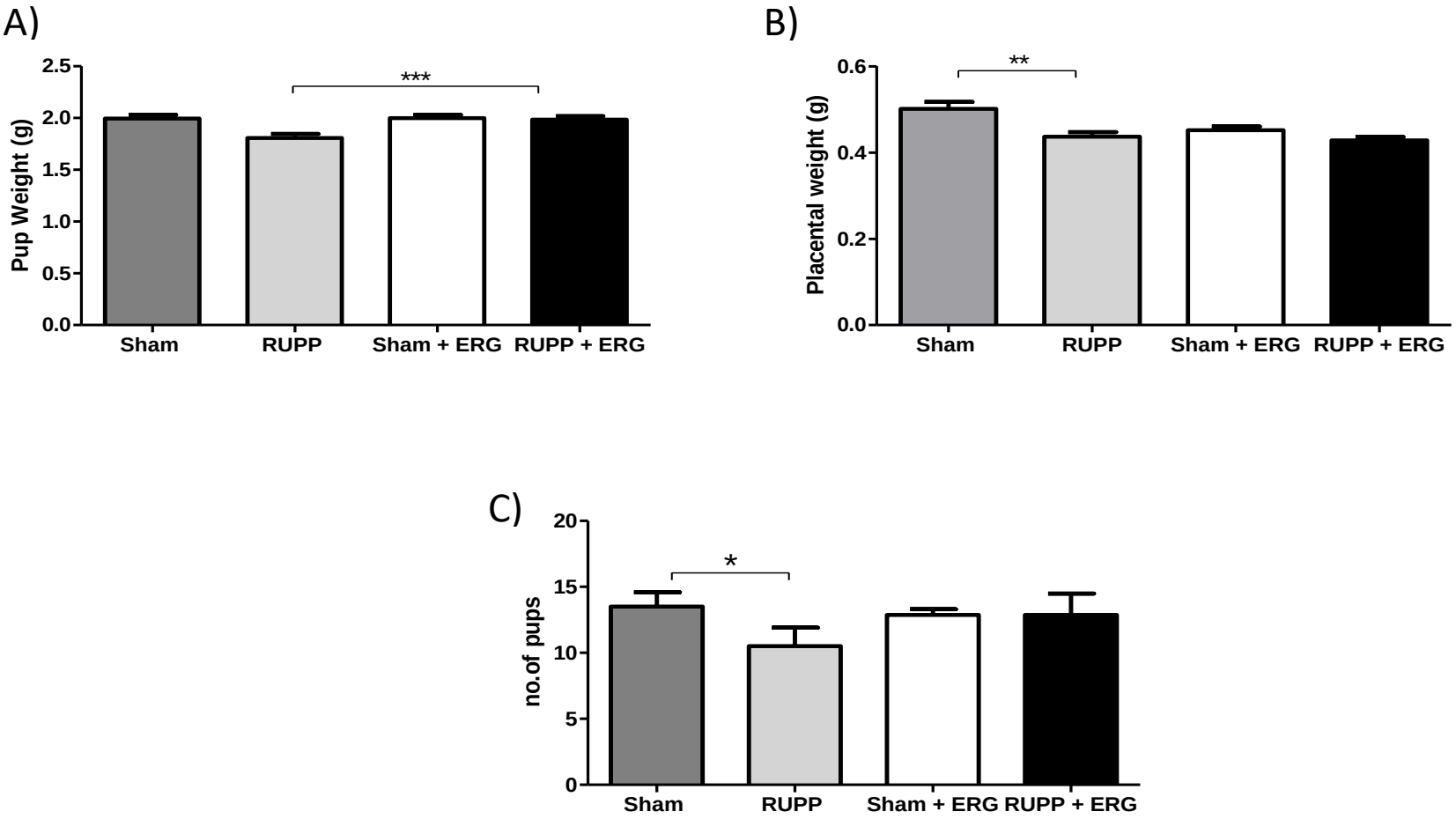
A)
Pup Weight (g)
2.5
2.0
1.5
1.0
0.5
0.0
***
Sham
RUPP
Sham + ERG
RUPP + ERG
B)
Placental weight (g)
0.6
0.4
0.2
0.0
**
Sham
RUPP
Sham + ERG
RUPP + ERG
C)
no.of pups
20
15
10
5
0
*
Sham
RUPP
Sham + ERG
RUPP + ERG

Figure 5.3: L-ergothioneine, improves fetal outcomes in RUPP rats: RUPP rats (n=8) show reduced a) pup weight and b) placental weight and c) litter size compared with sham group (n=8). Administration of L-ergothioneine (25mg/kg/day) improved fetal outcome in RUPP rats. Data is presented as mean ± SEM, $P<0.05$.

5.4.3 Effect of L-ergothioneine on circulating sFlt-1

There was no significant increase in the circulating soluble anti-angiogenic mediator sFlt-1 in the RUPP group compared to the sham group (2245.18 ± 263.18 pg/ml v 1365.99 ± 345.73 pg/ml, P= 0.27) (Figure 5.4). Administration of ERG significantly decreased circulating sFlt-1levels in RUPP rats (1444.04 ± 220.61 pg/ml, P= 0.03; Figure 4).

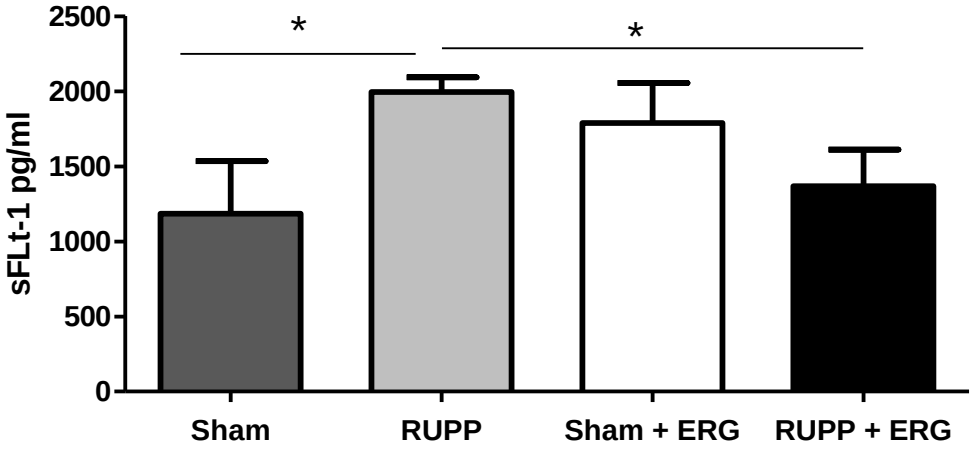

Figure 5.4: L-ergothioneine reduces circulating anti-angiogenic factor sFlt-1 in RUPP rats: Circulating levels of sFlt-1 were higher in RUPP rats (n=8) compared with sham controls (n=8). L-ergothioneine significantly reduced circulating sFlt-1 levels in RUPP rats. Data is presented as mean ± SEM, P<0.05.

5.4.4 Effect of L-ergothioneine on vasorelaxation in the RUPP model

Mesenteric arteries from the RUPP group displayed impaired vasorelaxation in response to bradykinin (BK) when compared to the sham group (R_{max}: 28 ± 7% versus 52 ± 9%; P= 0.01, log EC50: -6.62 ± 0.18 versus -6.77 ± 0.32 mol/L; P=0.67, Figure 5.5). However, no significant differences were seen in the vascular response to the endothelial independent vasodilator sodium nitroprusside (SNP) (R_{max}: 78 ± 5% versus 80 ± 4.5%; P= 0.36, log EC50: -7.09 ± 0.17 versus -7.17 ± 0.35 mol/L, P=0.85, Figure S3.A) or Acetycholine (ACh) (R_{max}: 83 ± 8% versus 81 ± 6%; P= 0.14, log EC50: -7.097 ± 0.15 versus -7.006 ± 0.10 mol/L, P= 0.62 Figure S.3B). Treatment with ERG had a significant effect on Ach (R_{max}: 83 ± 8% versus 71 ± 4%; P= 0.66, logEC50: -7.127 ±0.17 versus -6.809 ± 0.11, P= 0.04, Figure 5.10.SA) in RUPP rats. Treatment with ERG had no significant effect on the vasorelaxant responses of mesenteric vessels in response to BK (R_{max}: 23 ± 5% versus 28 ± 7%; P= 0.87, log EC50:-6.627 ± 0.18 versus -6.755 ± 0.21 mol/L, P=0.67, Figure 5.5), or SNP (R_{max}: 78 ± 5.7% versus 74 ± 4%; P= 0.76, log EC50: -7.079 ± 0.17 versus -7.145 ± 0.24, P=0.87, Figure 5.10.SB) in RUPP rats.

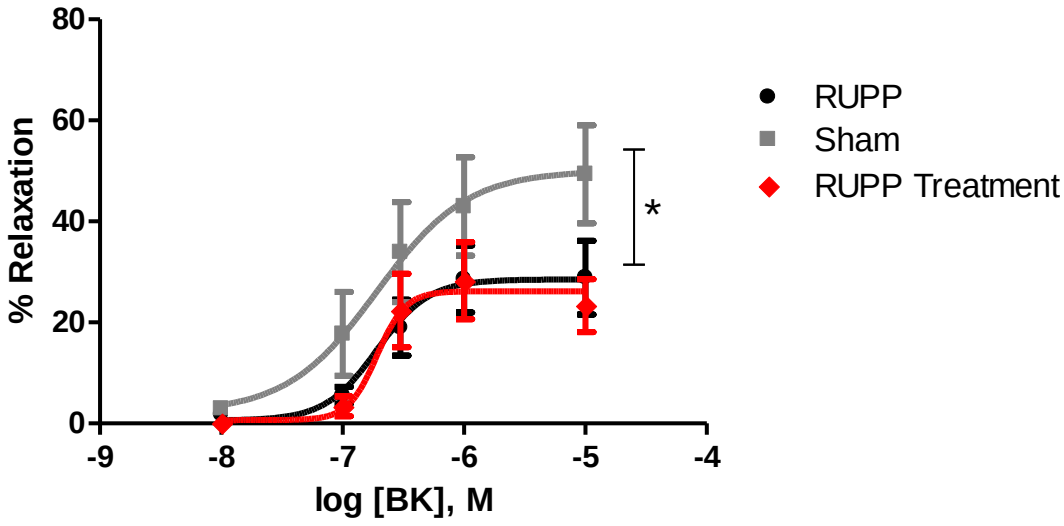

Figure 5.5: Impaired vasorelaxtion in mesenteric arteries in the RUPP model

RUPP rats: RUPP rats (n=8) showed significant impaired vasorelaxation to bradykinin compared to sham group. L-ergothioneine did not attenuate the impaired vasorelaxation to bradykinin compared to the RUPP group. Relaxation is calculated as a percentage of the maximum contraction and expressed as mean ± SEM. P<0.05.

5.4.5 L-ergothioneine alters placental expression of markers of mitochondrial-ROS detoxifying enzymes

Placental expression of mitochondrial orchestrator proliferator activated receptor γ co-activator 1-α (PGC-1α) (0.4 fold ± 0.2, P= 0.008, Figure 5.6A) and mitochondrial-ROS detoxifying enzymes including uncoupling protein 1 (UCP-1) (0.2 fold ± 0.5, P= 0.04) were significantly decreased in the RUPP group compared to sham group. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a transcriptional regulator of the mitochondrial antioxidant defence system and coactivated by PGC-1α was also significantly reduced in the RUPP group when compared with the sham group (0.4 fold ± 0.3, P= 0.001, Figure 5.6). Placental expression of SOD1 antioxidant was significantly increased (1.4 fold ± 0.4, P= 0.02; Figure 5.6B), while mitochondrial SOD2 antioxidant was significantly reduced (0.6 fold ± 0.3, P= 0.01, Figure 5.6B) in RUPP group compared with sham group.

ERG treatment significantly increased placental expression of PGC-1α (1.7 fold ± 0.2, P= 0.02), UCP-1 (4.9 fold ± 0.5, P= 0.001) and Nrf2 (1.58 fold ± 0.3, P= 0.02, Figure 5.6A) in RUPP rats. ERG treatment significantly increased placental expression of SOD2 in RUPP rats (1.5 fold ± 0.3, P= 0.01, Figure 5.6B). There was

no significant difference in placental expression of the ERG transporter SLC22A4 in any of the experimental groups studied Figure 5.6B).

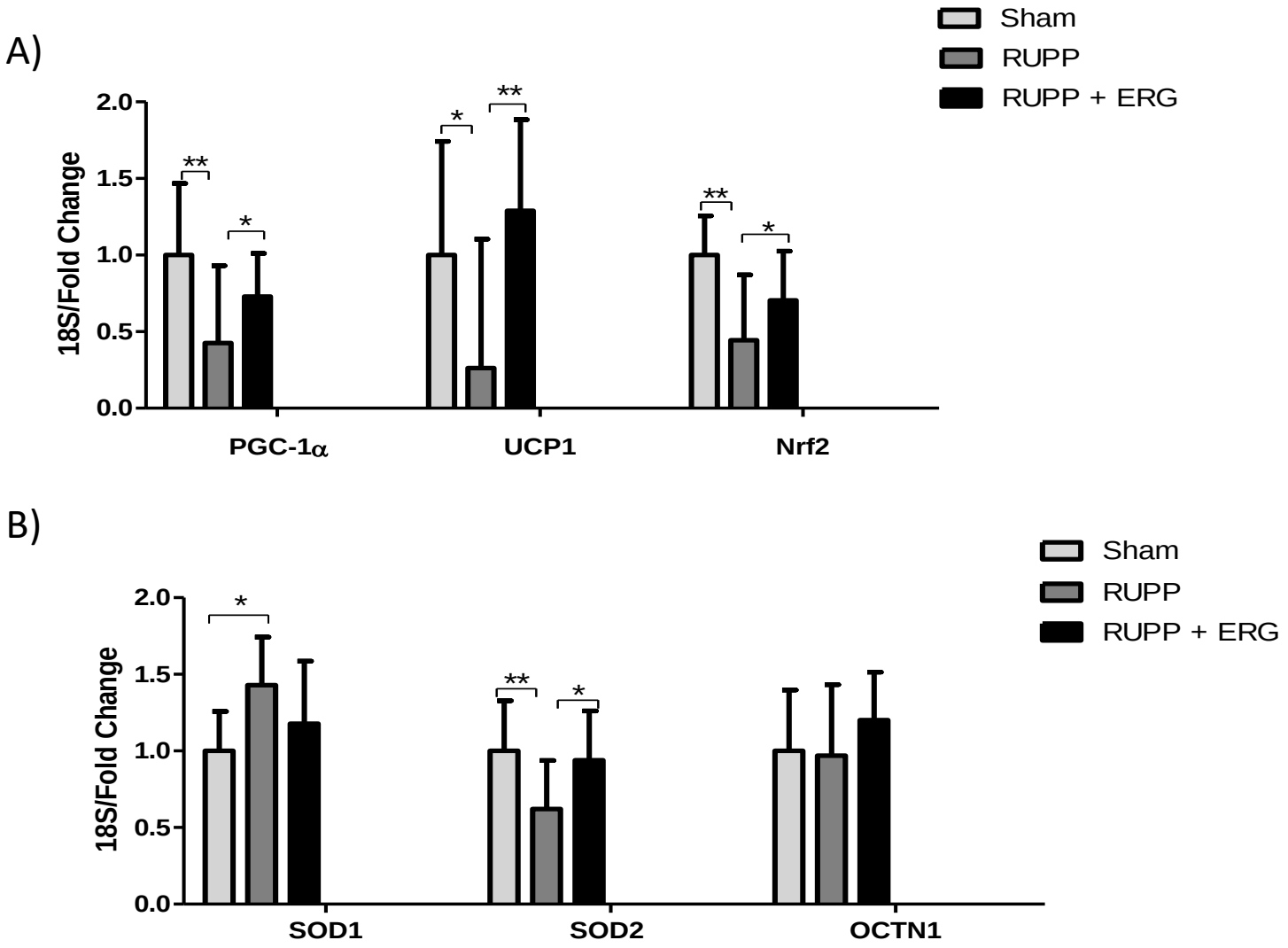
A)
Sham
RUPP
RUPP + ERG
18S/Fold Change
2.0
1.5
1.0
0.5
0.0
**
*
**
*
**
*
PGC-1α
UCP1
Nrf2
B)
Sham
RUPP
RUPP + ERG
18S/Fold Change
2.0
1.5
1.0
0.5
0.0
*
**
*
SOD1
SOD2
OCTN1

Figure 5.6: L-ergothioneine regulates placental expression of both markers of mitochondrial-ROS detoxifying enzymes in RUPP rats: a) Placental expression of PGC-1α, UCP-1 and Nrf2 expression were significantly reduced in RUPP rats (n=8) compared with sham group (n=8). L-ergothioneine significantly increased placental expression of all these mitochondrial markers in RUPP rats. b) Placental expression of antioxidant SOD1 was significantly increases while mitochondrial SOD2 expression was significantly decreased in the RUPP group compared to sham group. L-ergothioneine significantly increased mitochondrial antioxidant SOD2 expression in RUPP rats. SLC22A4 expression was not significantly different between any of the studied groups. Data are presented as mean ± SEM, $P<0.05$.

5.4.6 L-ergothioneine reduces mitochondrial-specific H_2O_2 in kidney tissue

MitoP/MitoB ratio showed an increasing trend in kidney tissue of the RUPP group compared with the Sham group (0.028 ± 0.001 v 0.024 ± 0.006; n=3, P=0.01, Figure 5.7), indicating for the first time that mitochondrial-specific H_2O_2 is increased *in vivo* as a result of placental ischaemia. Furthermore, pre-treatment with ERG significantly reduced the MitoP/MitoB ratio in kidney tissue in RUPP rats (0.024 ± 0.003 v 0.022 ± 0.001; n=3, P=0.05, Figure 5.7), establishing that L-ergothioneine reduces mitochondrial-specific H_2O_2 production *in vivo.*

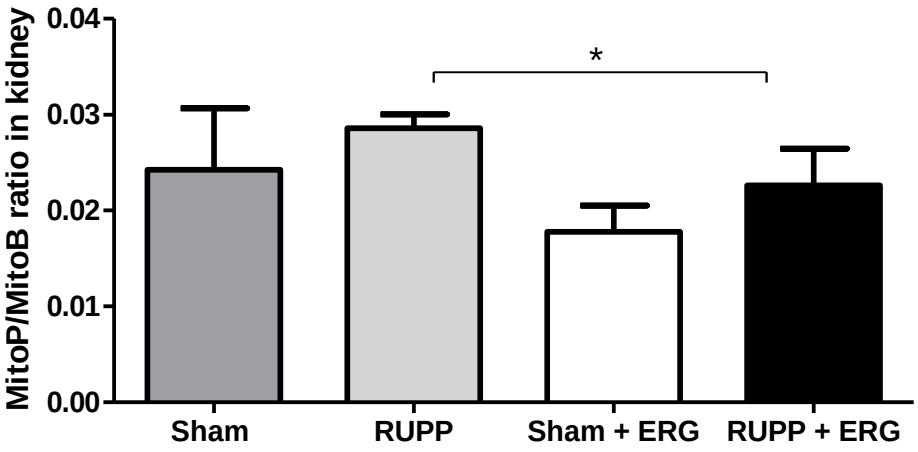

Figure 5.7: L-ergothioneine reduces mitochondrial-specific H_2O_2 levels

Rats were injected with MitoB for 4 hrs and the ratios of MitoP/MitoB were determined by LC/MS/MS. Data is represented as Mean ± SEM of 3 samples per group and corrected for ratio. Statistical analysis was determined by a two-tailed student t-test: * $P < 0.05$.

5.5 Discussion

Despite a significant amount of publications providing evidence for the pathological role of oxidative stress in the development of pre-eclampsia, clinical data from antioxidant vitamin trials have been negative (187, 365). One probable reason for these negative findings may be due to the fact these interventions missed the primary intracellular producer of ROS, namely the mitochondria; or because in the presence of free iron they are actually pro-oxidant (330). In the present study, L-ergothioneine, an amino acid with potent antioxidant properties, attenuated hypertension and rescued fetal growth restriction in the preclinical RUPP rat model of pre-eclampsia, which closely mimics many aspects of pre-eclampsia during pregnancy. Additionally, this study also reported the novel finding of reduced mitochondrial H_2O_2 levels *in vivo* following L-ergothioneine administration. This work has established L-ergothioneine as a mitochondrial-targeted antioxidant, which ameliorated a number of phenotypic features of pre-eclampsia in a preclinical model of disease, mediated in part by the reduction of mitochondrial-ROS, and subsequently highlighted its potential as a viable therapeutic for the treatment of pre-eclampsia.

L-ergothioneine is a water-soluble amino acid that is derived entirely from dietary sources. It has garnered much attention recently, as a potential therapeutic intervention due to its influential antioxidant resources, in addition to its preferential accumulation within tissues undergoing significant oxidative stress. A recent study investigating the reproductive safety profile of ERG in pregnant Sprague Dawley rats, established that ERG was well tolerated and with no adverse effects on a number of parameters (number of mating days, gestation length, pup viability index or litter parameters) (231). Furthermore, ERG treatment of diabetic rats significantly

improved embryo formation and quality (366). Collectively these data suggest ERG treatment may be safe to use in pregnancy and may prevent embryo malformations mediated by oxidative stress early in pregnancy, hence we decided to administer ERG at an early point in pregnancy (GD11) in the preclinical RUPP model of pre-eclampsia.

The therapeutic effects of ERG in response to induced tissue damage in models of ischemic-reperfusion injury in the liver (225) and intestine (360) have previously been reported by the dampening of markers of oxidative stress and inflammation. Our novel data using ERG reported a reduction in hypertension and an improvement in fetal weight in response to placental ischemia in the preclinical RUPP model of pre-eclampsia, in part due to regulation of mitochondrial-specific ROS. Recent evidence identified a prominent role for mitochondrial-specific ROS (mROS) in modulating hypertension (108)**.** Using two *in vivo* murine models of hypertension (Ang II-induced and DOCA salt), this group established that using a mitochondria-targeted antioxidant (Mito-Tempo) alleviated endothelial dysfunction, reduced vascular mitochondrial superoxide and subsequent hypertension (108). Furthermore, similar to our work, Vaka et al showed in the preclinical RUPP model that placental ischemia dysregulated mitochondrial function with elevated mROS and identified that treatment with mitochondrial targeted antioxidants attenuated hypertension with improvement in fetal outcomes in treated RUPP rats (204).

Deleterious circulating mediators including sFlt-1 are secreted in response to placental ischemia and have devastating consequences on the maternal vasculature. Furthermore, we previously detected increased levels of mitochondrial-specific superoxide production in HUVEC's incubated with plasma from women with pre-

eclampsia compared with matched controls and non-pregnant controls (109). Additionally, Zsengeller et al. established an inverse correlation between placental sFlt-1 and mitochondrial complex IV, suggesting that sFlt-1 may be harmful to mitochondria in pre-eclampsia (30). In our study, ERG treatment reduced the circulating levels of sFlt-1 in RUPP rats, indicating that ERG may preserve mitochondrial function in part by reducing the availability of sFlt-1 to induce mitochondrial damage. The reduction in sFlt-1 as a result of ERG treatment didn't result in a subsequent reduction in maternal vascular dysfunction in the RUPP rats.

The capacity to accurately measure the concentration of reactive oxygen species, in particular mitochondrial-specific ROS *in vivo* has proved to be extremely challenging yet is essential in understanding their physiological roles in certain disease entities. The detection of mitochondrial-specific ROS such as superoxide using fluorescent probes can be inferred, with certain caveats, specifically in regards to the difficulty of accurately quantifying fluorescence changes , or ex-vivo tissue measurements of ETC complexs, these methods can be less selective and sensitive due to excess processes and equally not physiologically relevant. One potential alternative is to use exogenous ratiometric probes (MitoB) injected into the animal models, that readily accumulate in mitochondria of tissues due to its triphenylphosphonium cation composition and which reacts with H_2O_2 *in vivo* to produce a diagnostic exomarker (MitoP) that can be accurately quantified by mass spectrometry (367).

In this study, we have shown for the first time that this method of measuring mitochondrial H_2O_2 production *in vivo* is applicable in a rat model, without any adverse effects on reproductive safety. Furthermore, we reported that mitochondrial

H_2O_2 was significantly increased in kidney tissue in the RUPP group compared with the Sham group. This is in agreement with recent work by Vaka et al, have shown an increase in mROS in kidney tissue in the RUPP group by examining ETC activity and cellular respiration ex-vivo using isolated mitochondria from kidney tissues (204). More importantly, treatment with ERG resulted in a significant reduction in mitochondrial-specific H_2O_2 production in kidney tissue in RUPP rats, indicating that ERG may be mediating its therapeutic effects in part by directly reducing mROS production in the preclinical model of pre-eclampsia. Further evidence to support our theory is provided by the fact that placental expression of markers of mitochondrial-ROS detoxifying enzymes and their transcriptional regulators were significantly improved in ERG treated RUPP rats in contrast to their reduced expression in RUPP rats.

At present, only one study has investigated the pharmacokinetics of ERG administration in human subjects (232). ERG was rapidly absorbed and retained within the tissue/plasma with relatively low urinary excretion (<4% of administered dose). This work was further extended by YiTang et al. who established that ERG and its metabolites are widely distributed in various tissues in male mice administered ERG over a range of days, this group also established that ERG was highly retained in the body and suggested this could be as a result of possible reabsorption by the kidneys (234).

This study provides evidence of the therapeutic potential of ERG in in a preclinical model of pre-eclampsia. ERG improved high blood pressure and fetal weight in the RUPP rat model of pre-eclampsia. Furthermore, ERG treatment significantly altered mitochondrial function in both the kidney and placental tissue which may in part be

responsible for the beneficial effects on phenotypic features of pre-eclampsia in the RUPP model. Given its favourable safety profile, it's long half-life and resistance to auto-oxidation and multiple mechanisms of action, not least its ability to regulate mitochondrial function, further studies are needed to explicitly define the protective mechanisms of ERG in treating pre-eclampsia.

5.6 Conclusion

The cause of pre-eclampsia has remained an enigma despite intense research efforts in recent decades. Consequently, effective interventions and treatments remain elusive and currently there is no effective treatment for pre-eclampsia. Overall, this thesis provides evidence that exaggerated mitochondrial-specific ROS plays a pathological role in the development of hypertension in response to placental ischemia in a preclinical rat model of pre-eclampsia. Additionally, this thesis demonstrates that ERG, a nutraceutical antioxidant that targets mitochondria and reduces mitochondrial-specific ROS opens a new avenue of investigations for new therapeutic options for pre-eclampsia.

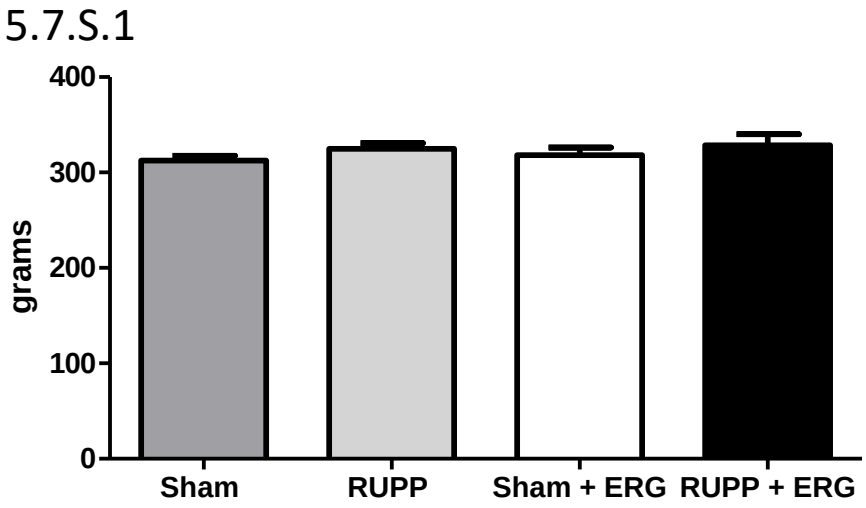

Figure 5.7 S.1: L-ergothioneine did not affect maternal weight in RUPP rats: Maternal weights did not change across all groups.

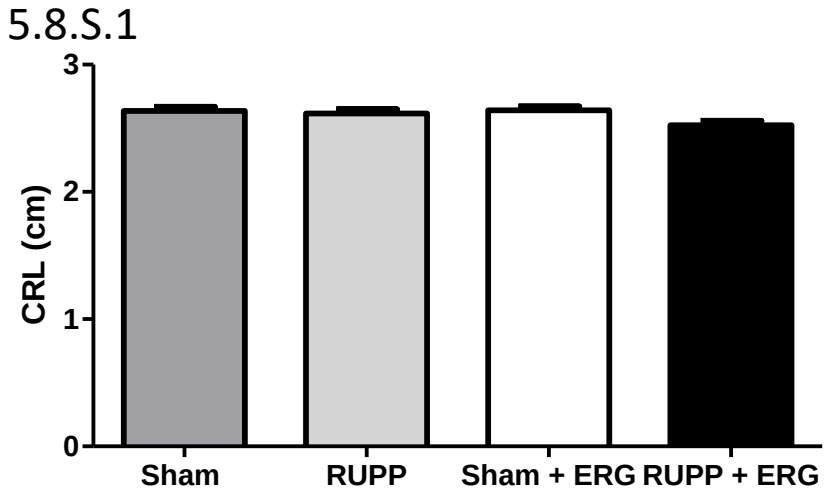

Figure 5.8 S.1: L-ergothioneine did not alter fetal crown to rump measurements in RUPP rats: Crown to rump measurements did not change across all groups.

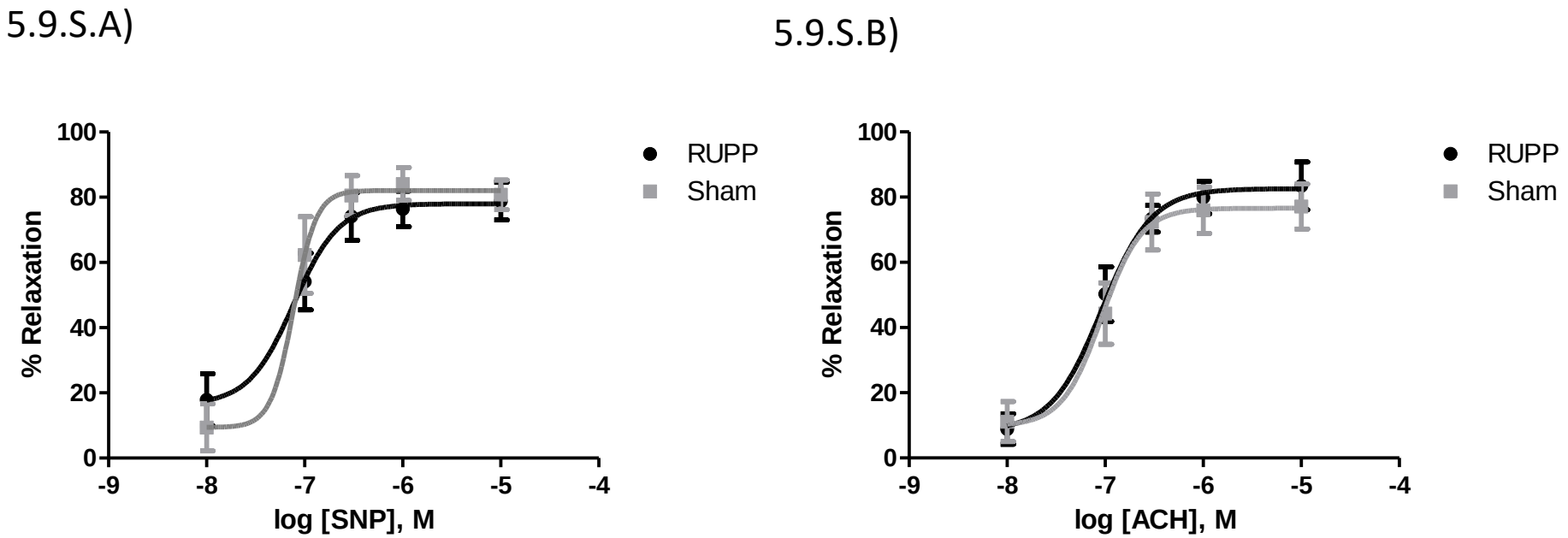

Figure 5.9.S: Vasorelaxtion in mesenteric arteries: RUPP rats (n=8) showed no vasorelaxation impairment to a) Sodium nitroprusside and b) Acetylcholine compared to sham group. Relaxation is calculated as a percentage of the maximum contraction and expressed as mean ± SEM. P<0.05

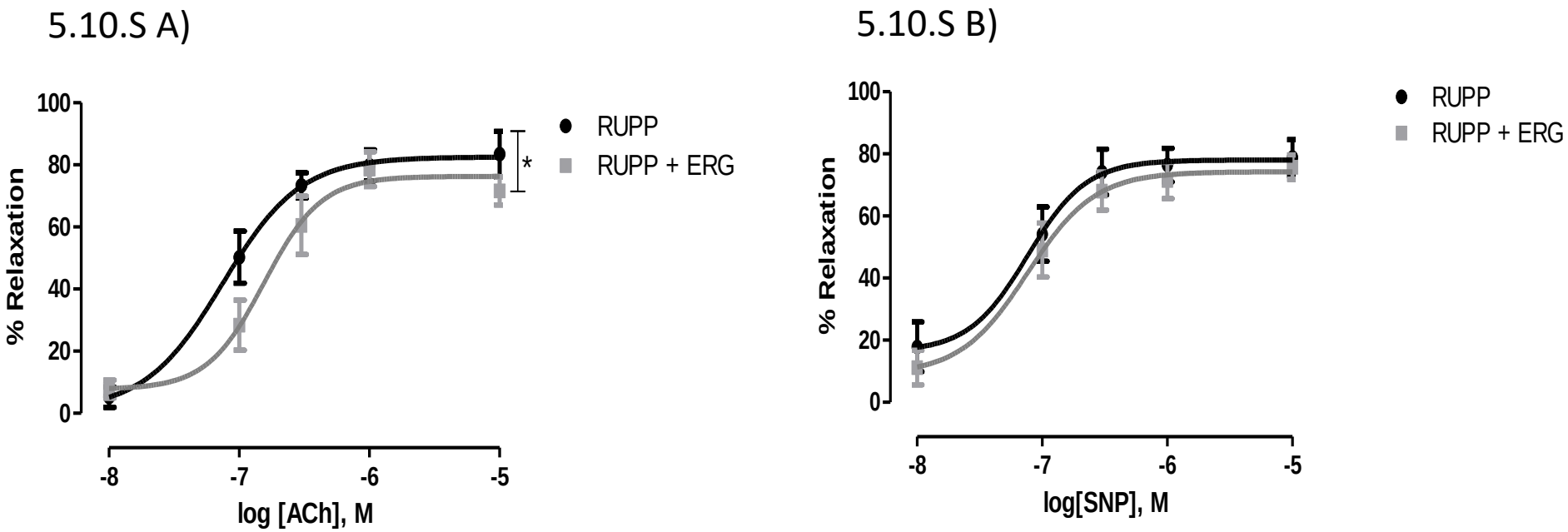

Figure 5.10.S: L-ergothioenine had no significant effect on endothelial dysfunction RUPP rats: L-ergothioneine treatment of RUPP rats (n=8) significantly decreased the impaired vasorelaxation to a) Acetylcholine and did not attenuate the impaired vasorelaxation to b) Sodium

nitroprusside compared to the RUPP group. Relaxation is calculated as a percentage of the maximum contraction and expressed as mean ± SEM. $P<0.05$

Chapter 6: Final Discussion

6.0 Chapter 6: Final Discussion

6.1 Summary of Results

This thesis focused on the pathophysiological role of mitochondrial dysfunction in pre-eclampsia and investigated the potential therapeutic potential of mitochondrial-targeted antioxidants, specifically L-ergothioneine for the treatment of this disorder. Pre-eclampsia is proposed to result from placental disease and progresses in two stages; abnormal placentation early in the first trimester subsequently followed by a maternal syndrome in the late second and third trimesters (38, 368), which consequently exposes the placenta to elevated levels of oxidative stress due to fluctuations in oxygen delivery. There is a growing body of evidence indicating that mitochondrial dysfunction acts as a pathogenic mediator of oxidative stress in pre-eclampsia and modulates the clinical characteristics of this syndrome (268, 271, 272). Mitochondria are the central cellular source of reactive oxygen species (ROS) and excessive production of mitochondrial-specific ROS is intrinsically linked to mitochondrial dysfunction (136, 267). During pre-eclampsia the compensatory properties regulating placental oxidative state are exhausted, leading to increased production of deleterious inflammatory factors and subsequent vascular dysfunction.

Mitochondrial DNA (mtDNA) are extremely vulnerable to damage due to their proximity to the region of ROS production and their deficiency of histone protection, and are often expelled into circulation as an indicator of mitochondrial dysfunction (89). There is genetic evidence which supports the involvement of mitochondrial dysfunction in mediating pre-eclampsia, as there was a high incidence of pre-eclampsia in families with pre-diagnosed mitochondrial dysfunction (271). Similarly, research has shown a positive association of mtDNA in maternal circulation with

increased risk of pre-eclampsia (268). Therefore, firstly I wanted to determine if mitochondrial dysfunction was evident in our cohort of 60 pre-eclampsia cases compared to 120 matched healthy controls from the SCOPE Cork cohort (Chapter 2). I showed that SOD antioxidant activity was significantly decreased at 15 weeks' gestation with a corresponding increase in ΔmtDNA copy number at 15 and 20 weeks' gestation in the pre-eclampsia cases compared to healthy matched controls. These findings highlighted that mitochondrial dysfunction has a role in the pathophysiology of pre-eclampsia.

The next step of this thesis was to determine if mitochondrial dysfunction evident in the pre-eclampsia cases of our study cohort provoked an innate inflammatory response (Chapter 3). In this chapter, I showed that TLR9 activity, a receptor for mtDNA, was significantly increased at time of disease in pre-eclampsia. Furthermore, I have shown a corresponding increase in production of neutrophil activation markers of innate inflammation particularly at the time of disease in pre-eclampsia. This provides evidence that circulating plasma mediators (including dysfunctional mitochondria) activate a TLR9-mediated innate immune response in pre-eclampsia.

Given that I have provided preliminary evidence that mitochondrial dysfunction has a potential pathophysiological role in pre-eclampsia, I wanted to test the hypothesis, that using a specific mitochondrial-targeted antioxidant, ERG, would alleviate mitochondrial-specific ROS generation evident in pre-eclampsia. To support this hypothesis, I initially performed *in vitro* experiments with a Jeg-3 trophoblast cell line pre-treated with ERG and subsequently exposed to an oxidative insult H_2O_2 and a number of mitochondrial parameters were measured (Chapter 4). In this chapter, I determined that pre-treatment with ERG had cytoprotective effects following

exposure to H_2O_2 insult. I showed ERG significantly reduced mitochondrial specific superoxide production, without altering mitochondrial mass. Furthermore, I described that ERG potentially mediated its protective effects via a PGC1-α regulated response involving increased expression of UCP-1 which may provide an alternative route to reducing exaggerated mROS generation and allied deleterious properties.

To further support the potential therapeutic role of ERG in dampening exaggerated oxidative stress and possibly mediating some of the clinical characteristics of pre-eclampsia, I wanted to examine its therapeutic effects in the Reduced Uterine Perfusion Pressure (RUPP) rat model, a preclinical *in vivo* model of pre-eclampsia (Chapter 5). Rats were administered ERG on GD 11 prior to RUPP surgery. In this study, I showed that ERG significantly reduced blood pressure in the ERG treated RUPP rats. Furthermore, I saw a significant increase in pup weight in the ERG treated RUPP rats. Interestingly, I provided novel evidence that ERG significantly dampened mitochondrial H_2O_2 production in vivo in kidney tissue using unique ratiometric exomarker probes. Additionally, I also provided evidence that ERG positively altered the placental expression profile of mitochondrial-ROS detoxifying enzymes and their mitochondrial transcriptional regulators in RUPP rats, similar to the changes I had seen in my *in vitro* placental model (Chapter 4).

This current thesis highlights that mitochondrial dysfunction has a role in the aetiology of pre-eclampsia by potentially provoking a neutrophil-driven innate immune response via TLR9 activation. Furthermore, I have shown that mitochondrial-targeted antioxidant ERG mediates its protective properties in *in vitro* and *in* vivo models of pre-eclampsia in part by directly educing mROS production with consequent amelioration of clinical characteristics of pre-eclampsia.

6.2 Implications of this work

This thesis has shown that mitochondrial dysfunction plays a pathophysiological role in pre-eclampsia by potentially activating a neutrophil-mediated innate immune response and that treatment of this disorder with specific mitochondrial-targeted antioxidants warrants future investigations. Pre-eclampsia was first considered to be a mitochondrial disorder in the 1980s (271). It was shown later that mitochondrial dysfunction in pre-eclampsia placentas induces oxidative stress (104). To date, there is evidence of disruptive homeostasis in factors regulating oxidative stress, with a decline in the antioxidant system and an increase in prooxidative ROS in women with pre-eclampsia (277, 289, 369). I provide evidence in this thesis that mitochondrial dysfunction is a significant contributor to this disruption. Mitochondrial DNA (mtDNA) encodes for 37 genes programmed by the mitochondrial genome (78) and is frequently used as a biomarker of mitochondrial dysfunction. mtDNA is particularly vulnerable to oxidative damage due to their intimate location in the electron transport chain (ETC) in the mitochondrial matrix and their lack of protective histones (282).

In the first study (Chapter 2), my results are in agreement with previous research investigating the role of mitochondrial dysfunction in pre-eclampsia (109, 268, 272, 294, 370). In Chapter 2, I investigated for evidence of mitochondrial dysfunction longitudinally in the SCOPE Cork cohort. In early gestation, mtDNA was not significantly different between cases and controls at 15 and 20 weeks' gestation. However, a significant increase in ΔmtDNA (15 and 20 week cases v 15 and 20 week controls) was evident. This increase in mtDNA early in gestation in women with pre-eclampsia correlated with the increased in mtDNA at time of disease in pre-eclampsia previously described by our research team (109). Upon longitudinal investigation, I

showed that mtDNA increased in cases as gestation progressed. The limitations to this study was the sample size for time of disease (n=25), even though there was a significant difference seen between cases and controls, the TOD cohort was smaller than 15 and 20 weeks (n=60) cohorts used in my investigations. Equally, in this thesis I used samples from the SCOPE Cork cohort, therefore it would be interesting for future studies to examine mtDNA in a larger multinational cohort to examine additional determinants of mtDNA levels such as ethnicity.

Oxidative stress results from an imbalance in production of ROS and antioxidant levels. SOD antioxidants play a critical role in mediating oxidative damage instigated by exaggerated superoxide generation in pre-eclampsia. In this thesis, I showed that there was a significant reduction in SOD activity at 15 weeks' gestation in pre-eclampsia. This result correlates with previous research where SOD levels at 10-14 weeks' and 20-24 weeks gestation were significantly lower in pre-eclampsia (291). When examining the point in gestation where the decrease in SOD activity was most evident in my cohort, my results propose that the increase in ΔmtDNA seen between 15-20 weeks in pre-eclampsia may be a consequence of reduced SOD antioxidant activity at 15 weeks which consequently increased vulnerability of mtDNA to oxidative damage.

There is circumstantial evidence that both exercise and healthy eating protect against mitochondrial diseases by maintaining mitochondrial function (297, 298), therefore, in this thesis, I wanted to examine if lifestyle and nutritional factors had an effect on the amount of mtDNA (mitochondrial dysfunction) in cases and controls throughout pregnancy. In this study, I showed that there both exercise and nutritional intake had no significant effect on mtDNA. This was in agreement with previous studies (268,

302), where no association between lifestyle and nutritional factors and pregnancy outcome, despite diet being suggested to play a possible role in the management of pre-eclampsia (301). Future research specifically focused on the role of lifestyle and nutritional factors in mediating mitochondrial function using additional biochemical markers would be of interest. In this current study I used retrospective data from the SCOPE cohort from women who self-reported on their exercise levels. One potential improvement to generate more quantitative correlations in future research could involve monitoring exercise in study participants using mobile monitoring devices such as the FitBit.

While highlighting that mitochondrial dysfunction plays a pathophysiological role in pre-eclampsia in Chapter 2 in my thesis, the next part of my thesis was to examine if mtDNA provokes an innate immune response in pre-eclampsia (Chapter 3). The innate immune system and its mediators particularly neutrophils, play a role in the aetiology of pre-eclampsia (129, 130, 371, 372). In healthy uncomplicated pregnancy, there is evidence of a control systemic inflammatory response (373). Neutrophils are activated during pregnancy, however they are further stimulated in pre-eclampsia (374). There is exciting evidence that dysfunctional mitochondria may provide a possible trigger in activating the innate immune system (136). It is thought that mtDNA may act as DAMPs and provoke an innate immune response by binding to TLR9 on the surface of immune cells particularly circulating neutrophils. In this thesis, I showed that there was a significant increase in TLR9 activity at time of disease in women with pre-eclampsia compared to matched controls. In agreement with my results, previous studies have shown an increase in TLR9 expression in both placental and PMBC from women with pre-eclampsia (140, 310).

I also showed there was a corresponding increase in the production of neutrophil activation markers, calprotectin, myeloperoxidase, MMP8 and pro-inflammatory cytokine IL-8 in pre-eclampsia compared with matched controls. In this thesis, I wanted to measure TLR9 activation throughout gestation in both cases and controls. Interestingly, TLR9 was only significantly activated at time of disease (late in gestation) which correlates with the significant increase in mtDNA at this point also in gestation. When examining markers of neutrophil activation and innate inflammation, it became evident that while the inflammatory system may be initially activated early in pregnancy, the systemic response is significantly heightened in the third trimester of pregnancy which is further supported by a number of recent studies investigating inflammatory cytokine profiles in women with pre-eclampsia (325, 326, 375). Access to neutrophil counts would have allowed me to directly correlate the results from neutrophil activation markers from both study cohorts, however, this was not possible in SCOPE study.

Having determined that mitochondrial dysfunction plays a pathogenic role in pre-eclampsia, I next sought to examine the possibility of directly targeting mitochondria by using specific antioxidants to see if they could ameliorate some of the deleterious effects of mitochondrial dysfunction and exaggerated oxidative stress. The antioxidant I used in my thesis was the mitochondrial-targeted antioxidant L-ergothioneine, in part due to its safety profile in humans and subsequent potential to rapidly translate to a clinical setting. To date, there has been extensive research showing that oxidative stress is a prime mediator in the pathophysiology of pre-eclampsia yet antioxidants have been overwhelmingly unsuccessful in the treatment of pre-eclampsia as evident from numerous large clinical trials (187, 347). This may be due to the fact that these vitamin antioxidants remain sequestered in the cytosol and fail to reach the

mitochondria, therefore restricting the capacity to alter the oxidative damage seen in pre-eclampsia. In Chapter 4, for the first time I showed ERG's potential mechanism in a model of oxidant stress in placental cells. In this chapter I presented novel data that ERG may be mediating its effects via increasing UCP-1 expression which induces mild coupling and subsequent dampening of inflated mROS production while highlighting its potential as a therapeutic intervention in pre-eclampsia.

In my final chapter (Chapter 5), I examined ERG's therapeutic potential in an *in vivo* preclinical model of pre-eclampsia. The RUPP model is most widely used model for the study of pre-eclampsia (240, 253, 376, 377). In this chapter, I showed that ERG ameliorated some of the clinical characteristics of pre-eclampsia, namely reduction in mean arterial blood pressure, while rescuing fetal growth restriction, in the RUPP model of pre-eclampsia. Interestingly, I also provided evidence that these therapeutic effects are mediated in part by directly dampening mitochondrial-ROS production. In terms of clinical relevance, ERG is an amino acid that is currently FDA approved and is available as a supplement. While ERG has been extensively examined *in vitro* and to a lesser extent in *in vivo* models, to date, only one study has investigated the pharmacokinetics of ERG administration in humans (232). Healthy human subjects were administered either a 5mg or 25mg dose every morning for one week with follow up of a further 28 days with no adverse effects observed. From this study, ERG was rapidly retained and absorbed within tissue and plasma as well as low urinary excretion. Furthermore, there was evidence of a reduction in markers of oxidative stress in the study groups. The wider implication of my research demonstrates that ERG, a nutraceutical antioxidant that directly targets mitochondria and reduces mitochondrial-specific ROS could be a new therapeutic avenue of investigation for the treatment of pre-eclampsia.

6.3 Strength and Limitations

There are many strengths to this thesis. Firstly, in Chapter 2 and Chapter 3, the primary strength of my thesis was the use of a well-defined pregnancy cohort of first-time mothers, which enabled a thorough investigation of the role of mitochondrial dysfunction in pre-eclampsia. Many aspects of this cohort have strengthened the work in chapter 2 and 3 of this thesis namely: Using a well characterised cohort allowed me to gain information on Demographic, biochemical, physiological and clinical data collected from each mother enrolled in the SCOPE study. Furthermore, a total population of 1774 women were recruited to SCOPE Ireland Study, therefore providing matched controls to the 60 women with pre-eclampsia. This is a significant strength to this thesis as I could examine mtDNA and inflammation is the same cohort of women longitudinally across 3 gestational time points. Upon examining the literature, previous studies examining mitochondrial dysfunction in pre-eclampsia, the cohort sizes were very limited. In my thesis, I examine mitochondrial dysfunction longitudinally throughout pregnancy, whereas, the vast majority of the literature focuses on the third trimester when investigating mtDNA and the innate immune system in pre-eclampsia. Throughout my thesis, I have provided strong evidence for the role of mitochondrial dysfunction in a human cohort of pre-eclampsia and that directly targeting mitochondria with ERG using both *in vitro* and an *in vivo* model, I showed alleviation of oxidative stress and phenotypic features of pre-eclampsia. Finally, using the RUPP rat model of pre-eclampsia equally strengthened my thesis due to the number of phenotypic features of human pre-eclampsia that are evident in this preclinical model of disease. This thesis is the first time mROS was examined *in vivo* in the rat model. This is a novel technique to examine real time mROS is a massive strength to this area. Previous

research measuring mROS is done outside of the animal which alters and manipulates the tissue and therefore is not a true representation of mitochondrial stress. Here I showed for the first time, there is an increase in specific mROS in the kidney of the RUPP model and furthermore, treating this with Ergothioneine significantly reduced the mROS production. This also highlights that Ergothioneine is specifically acting on mitochondrial dysfunction strengthening my hypothesis.

Certain limitations are recognised. The study cohort in Chapter 2 and 3, while being a strength of my thesis, the use of samples from the SCOPE cohort collected outside of Cork, including UK, New Zealand, and Australia to generate a larger study cohort would be of benefit to validate my research outcomes. As well as this, in chapter 2 and chapter 3, more details could have been obtained from my patient cohort, specifically, how severe the pre-eclampsia outcome was for each of my women (such as the severity of the preterm pre-eclampsia cases). This could of gave an insight into whether there was a difference in the amount of mtDNA and inflammatory makers in the women who had an outcome of severe pre-term pre-eclampsia complications. Additionally, blood pressure measurements from the women at time of disease in my cohort, would have been a valuable addition in Chapter 2, table 2.1. When examining modifiable factors, in particular exercise, there is a clear limitation to this data and a definitive conclusion is hard to quantify. The data collected regarding exercise and diet was self-reported, therefore not a true representation of the data. To overcome this obstacle, this is further discussed in future work section 6.4. Furthermore, in Chapter 3, the initial experimental plan was to examine the inflammatory pathway early in pregnancy, however, on carrying out this experimental research, I showed that the inflammatory makers I was measuring were not activated early in pregnancy, therefore, the idea of looking at the

inflammatory response late in pregnancy was subsequently undertaken. Furthermore, it would have been beneficial to have the neutrophil count for the women who subsequently went on to develop pre-eclampsia to correlate with the results of the neutrophil activation markers. Additionally, having access to clinical data regarding the severity of cases within my case cohort, it would have been interesting to examine and correlate the increases seen within individual cases and the severity of the pregnancy outcome.

This PhD specifically looked at mitochondrial ROS and has illustrated the potential role of mROS plays in pre-eclampsia. However, there are other sources of ROS such as NADPH oxidase which plays a significant role in mediating oxidative stress. NADPH oxidases are activated by a variety of physiological and pathophysiological stimuli including inflammatory cytokines, mechanical forces and growth factors. While the mitochondria were the main focus as a source of ROS for this body of work, I acknowledge that there are many pathways involved in the role of oxidative stress, that may be worth examining in future work.

In this thesis, I have shown that Ergothioneine plays a role in potentially regulating mitochondrial function. However, I acknowledge that there is more research to be done regards to investigating mitochondrial dynamics as well as examining mitochondrial respiration as discussed in future work.

Finally, in Chapter 5, while animal models are excellent to examine the aetiology of a disease, the model will never fully replicate the human phenotype of pre-eclampsia.

6.4 Future work

It would be exciting to examine the role of mitochondrial dysfunction in the aetiology of pre-eclampsia in a larger study cohort. A recent phase II clinical trial, IMPROvED (IMproved Pregnancy Outcomes by Early Detection) was carried out in 7 European Centres as a validation study for biomarkers initially identified in the SCOPE study. The IMPROvED cohort could further validate my study findings investigating the role of mitochondrial dysfunction in pre-eclampsia using larger sample numbers.

It would be interesting to examine the effect of ERG on additional mitochondrial parameters including cellular respiration and oxygen consumption in the Jeg-3 placental cell line. This could be achieved using the Agilent Seahorse technology and would strengthen my existing body of work. Though the focus of ERG is as a potential mitochondrial antioxidant in this body of work, the many sources of ROS are interlinked in some shape or form therefore, future research could potentially examine the effects of ERG on different sources of ROS and different pathways.

Throughout this body of work, I have highlighted that ERG is a mitochondrial specific targeted antioxidant. I have provided evidence of this in Chapter 4 by highlighting its role in regulating mitochondrial function and furthermore in Chapter 5, by showing ERG reduces specific mROS in the kidneys in an *in vivo* model. However, future work is warrened to further elucidate its specific role in mROS. To further prove ERG is a mitochondrial specific antioxidant, carrying out an experiment to extract the mitochondria from treated cells would add to this body of work and illustrate that ERG gets into the mitochondria, therefore strengthening my hypothesis.

In this thesis, I have shown that TLR9 activation of the innate immune system may play a role in the pathophysiology of pre-eclampsia in late gestation. However, it would be exciting to examine the potential signalling pathways involved in mtDNA and TLR9 activation. Mitogen-activated protein kinase (MAPK) pathway is involved in oxidative stress, and DNA damage, therefore would be a potential pathway to further elucidate our hypothesis that mitochondrial dysfunction activates TLR9 signal and effects neutrophil downstream markers instigating an inflammatory response seen in late pre-eclampsia. Furthermore, an exciting avenue of investigation would be to elucidate how mtDNA triggers TLR9 activation. This would involve isolating placental mitochondria and extracting DNA from these mitochondria. DNA could be extracted from patients with pre-eclampsia and uncomplicated pregnancies and then used to treat TLR specific reporter cells to show that mtDNA contained within maternal plasma is the primary mediator of TLR9 activation.

To date, clinical trials in pregnancy have been very limited. This is due to the unknown effect a drug would have on the unborn foetus. Furthermore, the VIP trial, examining the role of antioxidants in pregnancy, highlighted a secondary outcome of reduced fetal weight in mothers that received Vitamin C. This trial highlights the challenges of treating pregnant women. Even though the *in vitro* data supporting the use of vitamin C as a potential therapeutic for pre-eclampsia to date it has proven to be very challenging to determine the optimal equilibrium between reducing oxidative stress while maintaining physiologically important redox signalling when administering antioxidant treatment *in vivo.* There has been no clinical trial investigating the use of ERG in pregnancy , however, there was one study examining ERG in pregnant rats and reported no adverse effects (231). To further add to the intergenerational effect of antioxidant treatment it would be interesting to examine

the offspring from a pre-eclampsia induced rat with and without ERG treatment to see if there was any adverse effect in pup outcome throughout life. In Chapter 5 I have shown that pup weight was increased in the ERG treated group, which is already an exciting start.

Finally, the RUPP model is a very good preclinical model for examining the maternal phenotype of pre-eclampsia and for the development of future novel therapeutics to treat this obstetric complication. In Chapter 5, ERG was administered on GD11, 3 days prior to RUPP surgery which induces the pre-eclampsia phenotype. However, most women who attend antenatal clinics present with some symptoms of pre-eclampsia. Hence future investigation of the translational impact of this novel antioxidant should involve beginning treatment with ERG on GD14, immediately after RUPP surgery. Given the fact that ERG has received FDA approval and is safe for human consumption, it makes the transition to future human clinical studies a very achievable target, however further pre-clinical investigations are warranted before this novel mitochondrial-targeted antioxidant reaches the clinic.

7.0 Conclusion

This thesis has provided evidence of mitochondrial dysfunction in women who develop pre-eclampsia. Additionally, this work has shown that mitochondrial dysfunction activates a neutrophil-driven innate immune response in women with pre-eclampsia which is mediated in part by activation of the TLR9 receptor. Furthermore, this novel work established that L-ergothioneine, a unique antioxidant directly targets the mitochondria and reduces exaggerated mitochondrial specific ROS production in both *in vitro* and *in vivo* models of pre-eclampsia. Additionally, in the *in vivo* model of pre-eclampsia, treatment with L-ergothioneine positively modulates some of the detrimental clinical characteristics evident in women with pre-eclampsia, highlighting the therapeutic potential of this mitochondrial-targeted antioxidant in the treatment of pre-eclampsia.

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Appendix C: Published Manuscripts

Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health 8 (2017) 1–5

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Review article

Oxidative stress in pre-eclampsia; have we been looking in the wrong place?

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1. Introduction

Pre-eclampsia is a disorder of late pregnancy. It is a major cause of maternal and perinatal morbidity and mortality, accounting for nearly 18% of all maternal deaths worldwide; an estimated 77,000 maternal deaths per year [1]. Poor placentation is considered to be an initial cause of the placental ischemia [2]. Placental ischemia in turn gives rise to oxidative stress in the placenta and leads to shedding of syncytiotrophoblast debris into the maternal circulation provoking a systemic maternal inflammatory response and release of sFLT and sENG causing maternal vascular endothelial dysfunction. The ubiquitous nature of the maternal vascular endothelium accounts for the diverse multi-system nature of pre-eclampsia.

Currently there is no treatment for pre-eclampsia except delivery of the placenta and the baby, with the attendant risk of iatrogenic prematurity and significant neonatal morbidity and mortality. As a result, intensive research endeavours have focused on defining the molecular mechanisms of pre-eclampsia and the identification of new pre-symptomatic biomarkers of the condition. This review focuses on the role of elevated oxidative stress in the pathology of pre-eclampsia and potential therapeutic agents targeting oxidative stress that may prevent or ameliorate this disorder.

2. Reactive oxygen species (ROS)

Reactive oxygen species are typically recognised as two groups; free radicals and non-radical products. Free radicals include superoxide (O_2^-), the hydroxyl radical (HO), lipid peroxy-radicals (LOO) and alkoxy-radicals (LO). Non-radical derivatives are more stable with a longer half-life, however, they can have strong oxidant properties. Non-radicals include hydrogen peroxide (H_2O_2), peroxynitrite (ONOO−) and hypochlorous acid (HOCl−) [3]. O_2^- acts as an oxidant as well as a reductant in biological systems [4]. O_2^- serves as an origin for many other biologically relevant ROS including H_2O_2, HO radical and ONOO− [3]. H_2O_2 is created by dismutation of O_2^-, this molecule can occur spontaneously or it can be promoted by superoxide dismutase (SOD). Hydroxyl radical (HO) is created when O_2 donates one electron to H_2O_2 in a reaction known as the Haber Weiss Reaction. The HO molecule is a highly reactive oxidant that attacks a variety of biomolecules such as lipids, proteins and DNA. Peroxynitrite (OONO−) results from the spontaneous reaction between O_2 and NO. OONO− is known to be a very strong oxidant and it can react with lipids, DNA and proteins [3]. This molecule reacts and modifies proteins and other cellular structures inflicting oxidative damage on these molecules.

3. Sources of ROS

Sources of ROS include NADPH Oxidase, xanthine oxidase and the mitochondria. NADPH oxidases are activated by a variety of physiological and pathophysiological stimuli including inflammatory cytokines, mechanical forces and growth factors. Xanthine oxidoreductase exists in two forms; xanthine oxidase and xanthine dehydrogenase. Xanthine oxidase transfers electrons to oxygen from NADH and uric acid and generates O_2^- and H_2O_2, whereas xanthine dehydrogenase transfers the electrons from hypoxanthine and xanthine to NAD^+ generating NADH and uric acid [3]. When a critical cysteine residue is oxidised by peroxynitrite, xanthine dehydrogenase (XDH) is converted to xanthine oxidase. Xanthine oxidase has been shown to contribute to experimental hypertension in animal models, however currently there is limited evidence supporting the role of xanthine oxidase in human hypertension [5]. Mitochondria are responsible for ATP production in the cell through oxidative phosphorylation. The electron transport chain involves NADH and flavinadenine dinucleotide (FADH) which act as electron transporters. Electrons go through four stages and finally electrons are transferred to an oxygen molecule. During normal mitochondrial function, electrons transfer from one complex to the next efficiently and there is minimal loss or leakage from the electron transport chain. However, oxidative stress is inherently linked to mitochondrial dysfunction due to disruption in the electron transfer between acceptors during oxidative phosphorylation in the inner mitochondrial membrane resulting in electron leakage.

The natural defence against ROS comprises both enzymatic and non-enzymatic systems. Superoxide dismutase (SOD) is a well-known antioxidant enzyme that prevents free radical mediated injury *in vivo* and *in vitro* by metabolising superoxide anions that are known to damage human tissues [6]. SOD is categorised into three isoforms, cytosolic CU, Zn-SOD, and mitochondrial Mn-SOD,

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which catalyse the dismutation of O_2^- into H_2O_2 and O_2. A decrease in the endogenous SOD antioxidant defence systems weakens the normal metabolic removal of O_2. Additionally, endogenous protein alpha-1-microglobulin acts as a radical scavenger and an antioxidant. It provides protection to tissue and protects the mitochondria from oxidative damage. Non-enzymatic antioxidants act as ROS scavengers and include vitamins A, C and E; glutathione, bilirubin and uric acid. Vitamin E is a fat-soluble vitamin and its main role is to protect against lipid peroxidation. Vitamin C scavenges free radicals in aqueous solution.

4. Oxidative stress and pre-eclampsia

Oxidative stress is an imbalance between the production of ROS and antioxidant defences [3], resulting in increased levels of ROS with resultant damage of cellular components including DNA, proteins and lipids. Normal pregnancy is characterised by a low grade oxidative stress; there are increased circulating levels of oxidised low-density lipoproteins and a reduction in total antioxidant capacity in pregnant women when compared with non-pregnant women [7]. Excessive oxidative stress is generally thought to be involved in the pathology of many pregnancy-related disorders such as fetal growth restriction (FGR), pre-eclampsia and miscarriage. Dysfunctional placentation is proposed to provoke a hypoxic reperfusion injury causing elevated oxidative stress in pre-eclampsia. By 10–12 weeks' gestation in normal pregnancy maternal blood flow in the placenta causes a local increase in oxygen and elevation in the activity of the antioxidant enzymes [8]. However, in pregnancies subsequently complicated by pre-eclampsia there is a decrease in antioxidant enzyme activity at the same gestation [9].

ROS are generally unstable and have a very short half-life, therefore accurately assessing O_2^- and H_2O_2 in the clinical setting is difficult. To address this, methods have been developed to measure stable markers of ROS that reflect an oxidative stress status. Biomarkers of oxidative stress in human samples include serum lipid hydroperoxides, plasma malondialdehyde (MDA) or urine F2-isoprostanes and uric acid [10]. Lipid peroxidation involves polyunsaturated fatty acids, including phospholipids, glycolipids and cholesterol that are vulnerable targets of oxidation. Increased lipid peroxidation plays a vital role in the pathology of pre-eclampsia by provoking endothelial dysfunction. MDA levels have been found to be significantly higher in pre-eclampsia in comparison with uncomplicated pregnancy [7]. Additionally, antioxidant SOD levels were significantly lower in pre-eclampsia in this study [7]. F2-isoprostanes are prostaglandin-like compounds produced *in vivo* by free radical induced peroxidation and are measured in human tissues and biological fluids [11]. In women with pre-eclampsia, isoprostanes have been shown to be elevated in the placenta [12]. Uric acid is the end product of purine degradation catalysed by the enzyme xanthine dehydrogenase and xanthine oxidase and its production is increased in oxidative stress [13]. Hyperuricemia in pre-eclampsia was thought to result exclusively from reduced renal clearance, however levels of uric acid are now also believed to increase by trophoblast breakdown, cytokine release and ischemia.

In recent years, overproduction of cell-free fetal haemoglobin (HbF) has been implicated as a new pathological factor of pre-eclampsia [14]. Haemoglobin reacts spontaneously with oxygen generating free oxygen radicals. Haemoglobin and its degradation products are toxic and can lead to oxidative stress in the maternal circulation [15]. A recent study of 433 women in early pregnancy, 86 of whom developed pre-eclampsia measured serum HbF levels from the first trimester and reported that the mean concentration of HbF in the women who went on to develop pre-eclampsia was significantly higher than in the control group [16]. Research from our group demonstrated elevated levels of several haemoglobin related metabolites including heme, bilirubin and biliverdin which were shown to be all increased in pre-eclampsia patients [17]. The metabolite heme binds to nitric oxide causing vasoconstriction, which may be central in pre-eclampsia.

5. Targeting oxidative stress in pre-eclampsia

5.1. Vitamins

Vitamin C and E are exogenous antioxidants known to down-regulate NADPH oxidase, a major source of ROS in the vascular wall, and also up regulate eNOS, which leads to vasorelaxation and a reduction in blood pressure [18]. The therapeutic potential of these particular antioxidants in the prevention of conditions associated with oxidative stress is supported by an extensive evidence base comprising of experimental studies [19], observational studies [20] and small clinical studies [21]. However, in stark contrast, large scale appropriately powered randomised clinical trials (RCTs) have been overwhelmingly disappointing. The VIP trial was a RCT of vitamin C and vitamin E in 2410 women identified as being at an increased risk of pre-eclampsia [22]. This study assigned the women with 1000 mg vitamin C and 400 IU vitamin E or the matched placebo daily from the period of the second trimester of pregnancy until delivery. This trial concluded that supplementation with vitamin C and vitamin E did not prevent pre-eclampsia. However, a secondary outcome from this clinical trial showed that vitamin supplementation increased the rate of babies born with low birthweight [22]. Another multicentre, randomised, double-blinded trial was carried out on 10,154 nulliparous women looking at vitamin C and vitamin E to prevent complications in pregnancy [23]. The primary outcome for this clinical trial was severe pregnancy-associated hypertension or severe or mild hypertension with many clinical outcomes such as eclamptic seizures. From their data in this clinical trial, supplementation with vitamin C and E did not reduce the incidence of pregnancy-associated hypertension or pre-eclampsia in low risk nulliparous women [23]. In comparison with the VIP trial, this study did not find significance between group differences in the rates of low birth rate and stillbirth. DAPIT was a randomised placebo-controlled clinical trial in the UK and Northern Ireland from 2003–2008 assessing vitamin C and vitamin E supplementation for the prevention of pre-eclampsia in women with type 1 diabetes [24]. Women in this study were randomly allocated to receive 1000 mg of vitamin C and 400 IU vitamin E or matched placebo every-day from between 8 weeks' gestation and 22 weeks gestation until delivery. This study showed no significant differences between vitamins and the placebo groups for any of the maternal outcomes. However, plasma ascorbate concentrations for both vitamins were significantly higher in the women that took the vitamins in comparison to the placebo group at 26 weeks' gestation and 34 weeks' gestation with a low antioxidant status at baseline [24]. In summary, in contrast to a significant supportive pre-clinical research base, these large clinical trials suggest that antioxidant therapy is ineffective in the treatment of disorders such as pre-eclampsia.

5.2. Nitric oxide

Nitric oxide (NO) is a major endothelium-derived vasoactive mediator [25] which acts as a potent vasodilator regulating vascular tone and tissue blood flow, as well as inhibiting platelet aggregation and leucocyte adhesions on the endothelial surface [26]. NO is synthesised by NO synthase (NOS) and activates soluble guanylyl cyclase to convert guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), which is then degraded by

phosphodiesterase 5 (PDE5) to guanosine monophosphate (GMP). Interruption of both NO and its secondary messenger cGMP has been implicated in the pathophysiology of pre-eclampsia [27]. NO signalling cascade is important for the placental function and development of vascular network. The altered balance of NO and ROS are thought to play a critical role in the pathogenesis of pre-eclampsia [28]. NO causes dilation of the utero-placental arteries, which is essential for trophoblast invasion and remodelling of the endothelium [29]. From these findings, it was hypothesised that low levels of NO may contribute to impaired cytotrophoblast invasion that is seen in pre-eclampsia.

In recent years, NO has become a promising therapeutic target for cardiovascular diseases such as atherosclerosis that are associated with oxidative stress [30]. Many studies have shown that endothelium-derived NO acquires an atheroprotective effect. Research has shown that responses to nitroglycerine cause vasodilation in coronary arteries which indicates the smooth muscle is responsive to NO. Furthermore, studies have examined the possibility of reversing endothelial dysfunction by enhancing the release of NO from the endothelium by stimulation of NOS or by protecting NO from oxidative inactivation [31].

Sildenafil citrate is a cGMP specific phosphodiesterase inhibitor, which potentiates the action of NO by inhibiting the degradation of cGMP. Herraiz et al. examined perinatal outcome after administration of sildenafil in a pre-eclampsia model in rats [32]. Sildenafil was shown to restore normal values of blood pressure and reduce proteinuria in a rat model of pre-eclampsia. Similarly, sildenafil has been shown to reduce plasma levels of sFlt-1 and sEng, in pre-eclamptic (l-NAME induced) Sprague–Dawley rats [33]. These two studies show the potential of sildenafil as a protective therapy for pre-eclampsia. However, in a small placebo controlled RCT of sildenafil in women with established pre-eclampsia showed no significant difference in time from randomisation to delivery in the two groups. [34]. However, there was a small increase in birth weight in the babies born to women in the sildenafil arm. Sildenafil may work by increasing uteroplacental circulation and perfusion which improves gaseous and nutrient exchange, improving fetal growth.

In vitro, sildenafil modifies the function of myometrial arteries but not the omental and chorionic arteries in placental bed biopsies from women with pre-eclampsia [34]. Sildenafil may not work in the treatment of pre-eclampsia due to the omental and chorionic arteries not being NO responsive. However, the effect that was seen was greater in IUGR than PE, suggesting chorionic plate vessels might be more relevant to the pathophysiology of PE. There is evidence from *ex vivo* and animal models of growth restriction that sildenafil citrate increases average pup birth weight and improves uteroplacental blood flow [35]. As a result this drug is now being investigated in a randomised double blind, placebo-controlled clinical trial (the STRIDER study) to quantify the effects of administration of sildenafil on pregnancy outcome in severe early-onset FGR [36].

5.3. Selenium

Glutathione peroxidase (GPx) and thioredoxinreductase (ThxRed) are selenoproteins with a selenium atom (Se) integrated in their active site in the form of selenocysteine [37]. This integration is essential for complete catalytic activity and as a result the activity and expression of these proteins are extremely sensitive to the amount of selenium present [38]. *In vivo* studies have shown that rats develop a pre-eclampsia-like syndrome following withdrawal of selenium in their diet [39]. Serum concentrations of selenium, expression and activity levels of glutathione peroxidase in maternal and umbilical venous blood samples were significantly reduced [40]. However, selenium concentrations were further reduced in umbilical venous samples in pre-eclampsia patients, [40]. The functional role of selenoproteins was examined in relation to protecting trophoblast cells from oxidative stress by up-regulating the selenoproteins GPx and ThxRed [37]. Cells were treated with either H_2O_2, *t*-butyl $H_2O_2$and cumene-H_2O_2 respectively to induce oxidative stress. The study showed all 3 forms of peroxide induced cellular stress and this increase could be reversed by supplementation with both inorganic and organic forms of selenium. Watson et al., concluded that Se mediates its protective effects through up regulation of the antioxidant enzyme activity of GPx and ThxRed which in turn increases the trophoblast capacity to tolerate oxidative stress [37]. More recently, the effects of selenium on markers of risk of pre-eclampsia in pregnant women was examined in a pilot randomised, control trial [41]. 230 women were randomised and given either 60 μg/d (re-enriched yeast) or placebo from 12–14 weeks' gestation until delivery. Whole blood selenium concentrations were measured at both baseline and 35 weeks gestation and furthermore, plasma selenoproteins P (SEPP1) concentrations were examined at 35 weeks' gestation. This study showed that between 12 and 35 weeks gestation, whole blood selenium concentrations were increased significantly in the selenium treated women opposed to the placebo group. Furthermore, SEEP1 concentrations measured at 35 weeks were also significantly higher in the treatment group than the placebo group.

5.4. Lifestyle

Lifestyle modifications including exercise, weight loss, diet change and reduced salt intake improve endothelial function, protect against vascular disease, lower blood pressure and might also lead to reduced vascular complications in patients with hypertension [42]. Prolonged exercise activates the Nrf2 pathway leading to the up-regulation of endogenous antioxidants including SOD, glutathione and peroxiredoxin [43]. However, the evidence to support advice on exercise for pregnant women with hypertensive disorders in pregnancy is limited. According to the American College of Obstetrics and Gynaecology (ACOG), pre-eclampsia is an absolute contraindication to aerobic exercise in pregnancy [44] as opposed to RCOG which advises caution while exercising with medical disorders in pregnancy [45]. Studies which have investigated whether exercise in pregnancy reduces the risk of pre-eclampsia, analysis suggests a reduced risk of preeclampsia with increasing levels of physical activity before pregnancy and during early pregnancy [46]. A recent study has shown a trend towards a protective effect of exercise and the occurrence of pre-eclampsia [47].

5.5. Mitochondria-targeted antioxidants

Mitochondria are the central cellular source of ROS. The placenta is a highly metabolic organ with vast numbers of functional mitochondria to manage the increasing demands of pregnancy. Mitochondrial dysfunction is a pathogenic mediator of oxidative stress and there is circumstantial evidence linking mitochondrial dysfunction with pre-eclampsia [48]. Research has shown elevated mitochondrial lipid peroxidation and increased evidence of susceptibility to oxidation in mitochondria of pre-eclamptic placentas [49]. Furthermore, increased activity of the placental mitochondrial electron transport chain in preterm pre-eclamptic patients compared to normotensive controls has been reported [50]. Alpha-1-mjcroglobulin (A1M) is a scavenger of free heme and radicals and protects cells against haemoglobin and heme induced oxidative damage. Increased levels of A1M have been reported in plasma and urine of women with pre-eclampsia [51]. Furthermore, A1M binds to the mitochondrial complex 1 subunit NDUFAB1 and

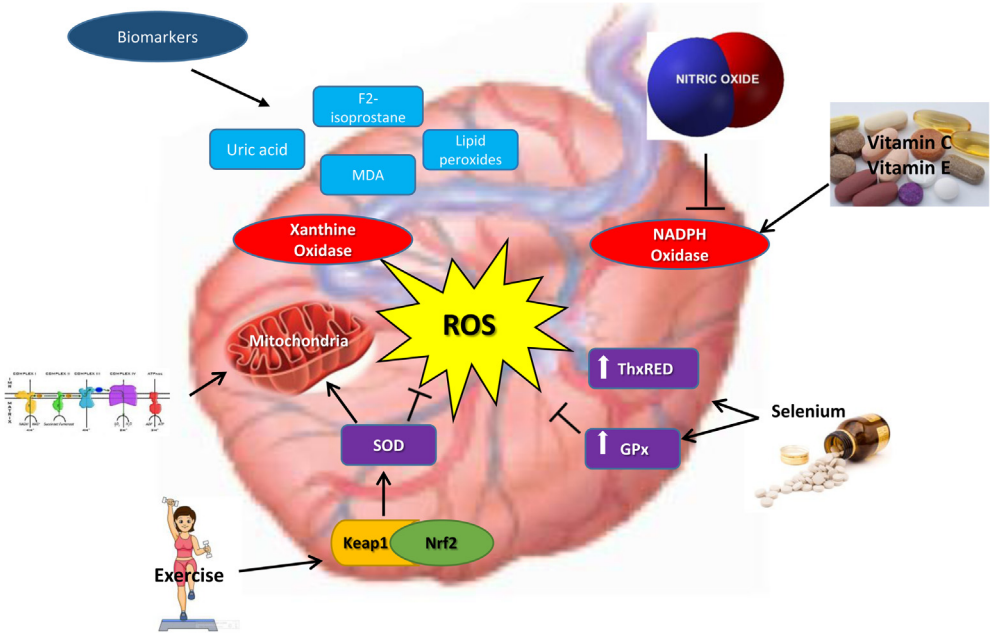

Fig. 1. Modulation of oxidative stress in pre-eclampsia. An imbalance in reactive oxygen species (ROS) is a cause of oxidative stress. Defective placentation is a common feature in pre-eclampsia which triggers a cascade of events including oxidative stress. Within the placenta, oxidative stress caused by unbalanced free radical formation from different sources of ROS including NADPH Oxidase, Xanthine Oxidase and the mitochondria. Antioxidants including SOD, GPx and ThxRed all target and inhibit ROS through different pathways and show a potential therapeutic effect on targeting oxidative stress in pre-eclampsia.

protects against mitochondrial swelling upon exposure to heme and ROS [52].

Recent work from our group demonstrated increased production of mitochondrial-specific superoxide in human umbilical vein endothelial cells (HUVEC) treated with pre-eclampsia plasma samples compared with normotensive controls [53]. The field of mitochondrial pharmacology has greatly progressed recently with a number of different pharmacology approaches in development, to tackle mitochondrial dysfunction. Interestingly, there is substantial evidence demonstrating that mitochondrial-targeted antioxidants may alleviate the clinical characteristics of pre-eclampsia. Recent work identified that mitochondria-targeted antioxidants (Mito-Tempo) alleviated endothelial dysfunction, increased vascular nitric oxide production, reduced mitochondrial superoxide and subsequent hypertension in two *in vivo* models of hypertension [54]. Mito-Tempo reduced mROS and increased vasodilation of visceral adipose arteries from morbidly obese subjects highlighting its potential to successfully treat pre-eclampsia in this obese cohort [55].

Based on the evidence discussed above (and summarised in Fig. 1), oxidative damage is a significant pathological mediator of pre-eclampsia and therefore it is critical to identify therapeutics targeting this process.

6. Conclusions

Oxidative stress occurs when the generation of ROS overpowers the fundamental antioxidant defences. In normal pregnancy the production of ROS increases towards the end of gestation, however antioxidant volume increases in order to maintain oxidative balance. In this review, we provided evidence of elevated oxidative stress and its role in the pathogenesis of pre-eclampsia. We also discuss novel antioxidant targets that warrant further investigation before entering the clinical arena as therapies. Future directions for potential treatments of pre-eclampsia should possibly target oxidative stress through mitochondrial-targeted antioxidants and selenium supplementation.

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Exploring the role of mitochondrial dysfunction in the pathophysiology of pre-eclampsia

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1. Introduction

Pre-eclampsia is a pregnancy disease that complicates 2–5% of pregnancies worldwide [1]. It is characterised by the development of hypertension and proteinuria after 20 weeks' gestation [2]. Pre-eclampsia is thought to occur secondary to abnormal placentation in early pregnancy [3] resulting from impaired placental trophoblast invasion and subsequent generation of an ischemic environment [4]. Placental ischemia is proposed to increase placental oxidative stress leading to the shedding of syncytiotrophoblast debris into the maternal circulation where it initiates a systemic maternal inflammatory response and subsequent endothelial dysfunction [5].

Oxidative stress is a cellular or physiological condition of elevated levels of reactive oxygen species (ROS) which damage cell structure and function. Antioxidant enzymes including superoxide dismutase (SOD) and glutathione peroxidase are components of the body's mechanism for combating oxidative stress. SOD is a powerful antioxidant that catalyses the reaction between two identical molecules of superoxide radical into oxygen or hydrogen peroxide. There is significant evidence that oxidative stress plays a role in the pathophysiology of pre-eclampsia [6,7]. Normal pregnancy is associated with an increase in oxidative stress due to a rise in maternal metabolism and maternal blood flow in the placenta by 10–12 weeks' gestation [8]. However, in pregnancies complicated by pre-eclampsia there is an exaggerated oxidative stress phenotype with a correspondent deficient antioxidant response.

Mitochondria are the dominant cellular source of ROS and there is strong evidence that mitochondrial ROS (mROS) play an important role in a variety of physiological processes including the regulation of cell differentiation, apoptosis, redox cell signalling and inflammation [9–11]. Furthermore, our research has implicated mitochondrial dysfunction as a potential mediator of oxidative stress in pre-eclampsia [10]. Cell-free DNA (cf-DNA) has been investigated as a universal diagnostic biomarker for a number of clinical applications, such as prenatal diagnosis and cancer monitoring [12,13]. Circulating cell free DNA is composed of both nuclear and mitochondrial DNA. Mitochondrial DNA (mtDNA) encodes for 37 genes programmed by the mitochondrial genome [14] and is often used as a biomarker of mitochondrial dysfunction. mtDNA are particularly vulnerable to oxidative damage due to its intimate location in the electron transport chain (ETC) in the mitochondrial matrix and its lack of protective histones [15]. More recently, there is emerging evidence suggesting that cell-free mtDNA (cf-mtDNA) is linked to disease progression such as, cardiovascular disease [16]. Circulating DNA in maternal plasma is mostly of maternal origin (hematopoietic and stromal derived) depending on gestational week and maternal bodyweight. Approximately 5–20% of the circulating DNA is derived from fetal/placental cells [17].

Our research has previously shown an increase in mtDNA in plasma samples at time of disease (TOD) in women with pre-eclampsia [18]. Hence the aim of this study was to characterise the role of mitochondrial dysfunction in women with pre-eclampsia compared to uncomplicated pregnancies by assessing levels of antioxidant enzyme superoxide dismutase and mtDNA at earlier time-points in pregnancy. We also examined if lifestyle and dietary factors affected mtDNA levels in pregnancy. We hypothesised that mitochondrial dysfunction plays a role in the pathogenesis of pre-eclampsia.

2. Methods

2.1. Study subjects

Subjects were recruited from the Screening for Pregnancy Endpoints (SCOPE) study Ireland which is an international multicentre prospective cohort study of nulliparous singleton pregnancies aimed to develop a screening test to predict adverse pregnancy outcomes including pre-eclampsia, SGA infants and spontaneous pre-term birth [19,20]. The clinical research ethics committee, University College Cork, approved the collection and use of samples for research purposes.

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A nested case-control study within SCOPE Ireland was conducted which included all pre-eclampsia cases in SCOPE Ireland and matched controls with a case-to-control ratio of 1:2. Pre-eclampsia cases were defined as women with systolic blood pressure ≥ 140 mm Hg and/or diastolic blood pressure ≥ 90 mm Hg on at least two occasions 4 hrs apart after 20 weeks' gestation and with proteinuria (24 h urinary protein ≥ 300 mg or urine dipstick protein ≥ +2). Randomly selected controls were taken from healthy pregnant women who had uncomplicated pregnancies which were defined as pregnancies not affected by pre-eclampsia, preterm birth or growth restriction and delivered at > 37 weeks. All blood pressure readings were < 140 and/or < 90 mmHg prior to the onset of labour. These were matched with the cases for maternal age, body mass index (BMI) and gestational age. Both 15 and 20 week samples were taken from the SCOPE study from women who subsequently went onto develop pre-eclampsia (n = 60) and controls (n = 120). Samples were also taken from a subset of women (n = 25) at the time of disease (TOD) with pre-eclampsia.

2.2. Superoxide dismutase enzyme activity

Superoxide dismutase activity was quantified in citrate plasma samples using a superoxide dismutase assay kit (Cayman chemical) which was used as per manufacturer's instructions. This assay utilizes tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. This assay measures a combination of activity from all three isoforms.

2.3. Sample collection and DNA extractions

Plasma samples were collected in BD Heparin Vacutainer tubes, placed on ice, and centrifuged at 2400*g* for 10 min at 4°C according to the standardised protocol. Plasma samples were stored at −80°C until analysis. Samples were analysed in a blinded manner. Total DNA was extracted from 200 µl of plasma from both controls and cases respectively with a QIAamp DNA mini kit (Qiagen). DNA was sonicated at 38 kHz ± 10% for 10 min to optimise DNA yield.

2.4. mtDNA quantification

Mitochondrial DNA was measured by real-time PCR using StepOne Plus Detection system using Taqman assays for mitochondrial DNA (hMitoF5, hMitoR5) [21]. Absolute quantification of the concentration of mitochondrial DNA (mtDNA) was determined by standard curve analysis and presented as copies/ml [21,22].

2.5. Maternal lifestyle factors

Women were asked at recruitment (15 ± 1 weeks of gestation) and at their second visit (20 ± 1 weeks of gestation) how many times each week did they carry out exercise that did not result in heavy breathing, which was the SCOPE definition of moderate-intensity exercise. The response was categorised as never, 1–3 times a week and daily. Similarly, the questionnaire administered at both time points asked women to report the frequency in which the consumed fruit and leafy vegetables. Scoring was similar to exercise, where the response was categorised as never, less than five pieces a week, and daily. Multivitamins were categorised into never, less than daily and daily.

2.6. Statistical analysis

Analysis was performed using GraphPad Prism and SPSS version 22 (SPSS Inc. Chicago, Illinois). Data were presented using median (± Interquartile range [IQR]) and comparisons of data between cases and controls were performed using a non-parametric Mann Whitney *U* test or Wilcoxon signed rank test as appropriate when data was not normally distributed. Data that was normally distributed were represented as mean (± SEM) and comparisons of data between cases and controls were performed using an unpaired *t*-test. P values < 0.05 were considered as statistically significant. Chi-squared test and the odds ratio (OR) was used to compare categorical variables.

3. Results:

3.1. Patient characteristics

There were 1774 participants in the SCOPE Ireland study. 68 (3.8%) women were diagnosed with pre-eclampsia and 60 were included in the nested case-control study with 120 participants selected as controls. The 60 women with pre-eclampsia were composed of 39 women who developed term pre-eclampsia and 21 preterm pre-eclampsia cases. As all cases and controls were matched nulliparous women, there was no significant differences observed between case-controls studies for maternal age, BMI, and gestational age at delivery. There was a significant difference in mean arterial blood pressure (MAP) in controls versus cases at both 15 and 20 weeks' gestation respectively (median [IQR]; 78.0 mmHg [73.33–83.33], n = 120, vs median [IQR]; 82.0 mmHg [75.0–87.66], n = 60, p = 0.0015 and media [IQR] 80.41 mmHg [75.3333–85.0], n = 120, vs median [IQR]; 83.5 mmHg [77.5–89.83] n = 60, p = 0.02). There was a significant difference in birthweight in controls compared to cases (3608.93 g ± 411.90 vs 2990.86 g ± 759.24; n = 120, n = 60; p < 0.0001). (Table 1).

3.2. Evidence of altered plasma SOD activity before pre-eclampsia

There was a statistically significant reduction in antioxidant SOD activity at 15 weeks' gestation between controls and cases (1.94 ng/ml ± 0.06 vs 1.69 ng/ml ± 0.06; n = 60, n = 119; p < 0.01; 95% CI; 0.04–0.45; Fig. 1A). There was no significant difference in SOD activity at 20 weeks' gestation between controls and cases, (0.82 ng/ml ± 0.02 vs 0.77 ng/ml ± 0.03; n = 119p = 0.21; 95% CI;

Table 1
Patient Characteristics in the study cohort.

	Preterm pre-eclampsia (n = 21)	Term pre-eclampsia (n = 39)	No pre-eclampsia (n = 120)
Mean Maternal age, years	31	29	29
Mean BMI	25	26	25
Maternal			
Mean Arterial Blood Pressure at 15 weeks	82.33 [74.16–86.33]	81.5 [74.58–88.0]	78 [73.33–83.33]
Mean Arterial Blood Pressure at 20 weeks	81.33 [77.66–89.66]	83 [76.08–87.0]	80.41 [75.33–85.0]
Fetal			
Mean Birth weight, g	2104	3300	3608
Mean gestational age at delivery	34	38	40
Perinatal death	1	1	0

Data are presented as mean or Median [IQR]. Mean Arterial blood pressure was calculated as MAP = (2 × diastolic) + systolic/3.

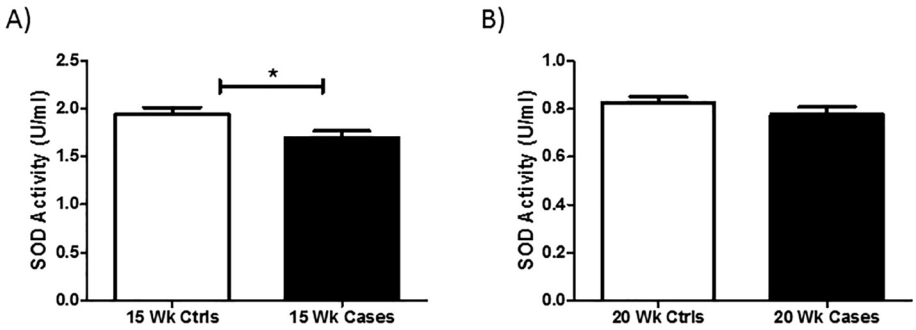

Fig. 1. A) SOD activity (U/ml) was significantly reduced at 15 weeks in cases compared to healthy controls (P < 0.01). B) SOD activity (U/ml) at 20 weeks was reduced in cases compared to healthy controls. Data are expressed as mean ± SEM.

−0.02–0.12; Fig. 1B).

3.3. Increased ΔmtDNA levels was evident between 15 and 20 weeks' before pre-eclampsia

There was no significant difference in the amount of total DNA between controls and cases at both 15 and 20 weeks' gestation (15 weeks: 7.06 ng/ml ± 4.08 vs 7.70 ng/ml ± 5.43 , n = 60n = 120, p = 0.38; 20 weeks 6.71 ± 3.26 vs 7.15 ± 3.44 , n = 60n = 120, p = 0.40; Fig. 2A). There was no significant difference in mtDNA at 15 weeks' gestation between controls and cases (median [IQR]: 2832.96 copies/ml [1711.17–5002.82] vs 2337.32 copies/ml [1357.11–5328.35], n = 58–117; p < 0.3381, Fig. 2B). Similarly, there was no significant difference in mtDNA at 20 weeks' gestation between controls and cases and (median [IQR]: 2885.57 copies/ml [1914.54–4834.12] vs 3307.7 copies/ml [1544.49–7396.92], n = 58–117; p < 0.7873, Fig. 2C). As pregnancy progressed the amount of mtDNA significantly increased in pre-eclampsia and healthy pregnancies (median [IQR]: 2337.32 copies/ml [1357.11–5328.35], 3307.7 copies/ml [1544.49–7396.92] and 6449.8 copies/ml [477.54–11145.9] n = 58 and n = 22.p < 0.0001, and median [IQR]: 2855.41 copies/ml [1740.19–5322.08], 2900.95 copies/ml [1958.36–5055.16], 5983.88 copies/ml [3209.67–16901.5] n-117 and n = 23. P = 0.009, Fig. 2D) at 15 weeks', 20 weeks' and TOD respectively.

However the mean difference in mtDNA between 15 weeks' and 20 weeks' gestation was significantly higher in cases compared with controls (2236 ± 796.0 copies/ml vs −555.3 ± 599.3 copies/ml mtDNA concentration in plasma, p = 0.0065; Fig. 2E).

3.4. Lifestyle and nutritional factors; effect of these factors on mtDNA

Moderate exercise had no significant impact on the amount of mtDNA in controls vs cases at 15 or 20 weeks' gestation OR 1.00; [CI 1.00–1.00 vs 1.00 CI [1.00–1.00] respectively (Table 2A). When assessing dietary factors such as leafy vegetable intake, fruit intake and multivitamin consumption, similarly there was no effect on the amount of mtDNA at 15 or 20 weeks' gestation in controls vs cases (Tables 3A, 3B, 3C).

4. Discussion

Mitochondrial dysfunction is a pathogenic mediator of oxidative stress in pre-eclampsia with elevated mitochondrial lipid peroxidation and increased vulnerability to oxidation evident in placental mitochondria in pregnancies complicated by pre-eclampsia [23]. In this present study we showed a significant reduction in antioxidant SOD activity at 15 weeks' gestation and an increase in the mean difference in

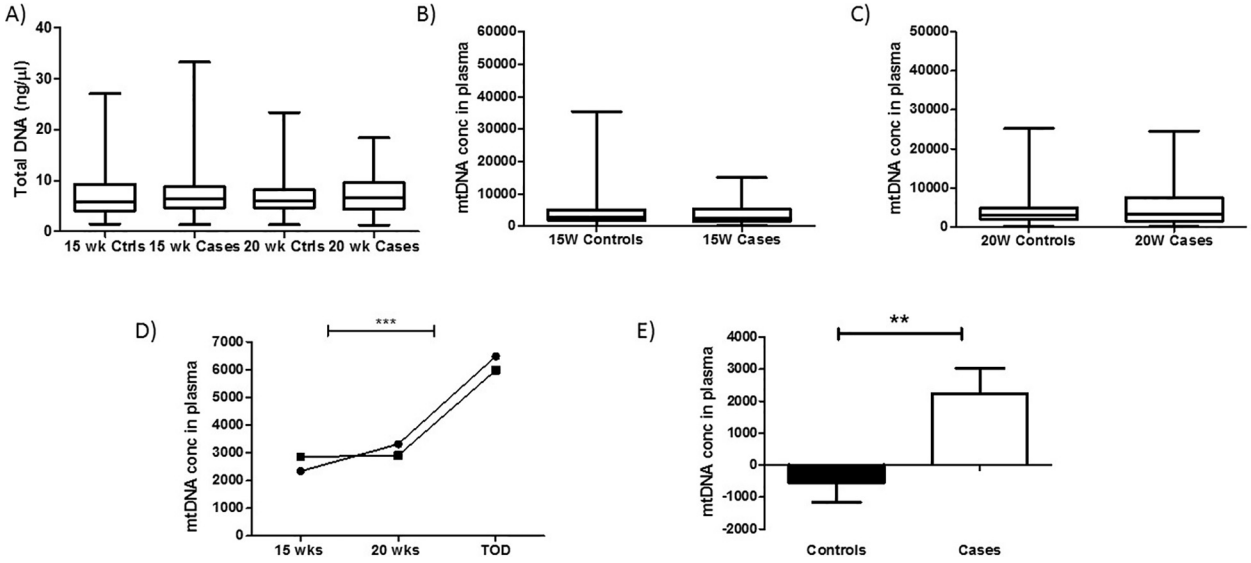

Fig. 2. A) Total DNA in maternal plasma in controls and cases showed no significant difference. B) mtDNA at 15 weeks showed no significant difference in controls when compared to cases C) mtDNA at 20 weeks was higher in controls compared to cases but not statistically significant D) Mitochondrial dysfunction is significantly increased as gestation progresses in pre-eclampsia and uncomplicated pregnancies (p < 0.001 and p = 0.0009) respectively.● = cases, ■ = control. Data represented as the median; [IQR]. E) Significant increase in the mean difference in mtDNA concentration between 15 and 20 weeks gestation in cases compared to controls (P < 0.01). Data represented as the mean; [SEM].

Table 2
The effect of lifestyle factors (Exercise) on mtDNA in cases compared to controls at 15 and 20 weeks' gestation.

Moderate exercise activity	N = 58	Case (mtDNA copy number/ml)	N = 117	Control (mtDNA (copy number/ml	OR	(95% CI)
Never exercised	19	1631.02 (815.92–3445.15)	23	2817.51 (1856.43–5602.91)	1.00	1.00–1.00
More than Once a week	31	2742.43 (1510.48–5328.35)	60	2676.89 (1669.87–3734.28)		
Daily	8	3928.08 (934.06–11509.39)	34	3070.21 (1912.75–5322.08)		
Never exercised	12	3242 (1367.75–9276.86)	20	2649.15 (1849.56–4454.55)	1.00	1.00–1.00
More than once a week	37	3249.79 (1493.10–7562.22)	67	2847.66 (1758.46–4248.59)		
Daily	9	4198.29 (1632.68–5398.94)	30	2790.07 (1998.00–5385.22)		

Table 3(A)
The effect of dietary factors (Fruit intake) on mtDNA in cases compared to controls at 15 and 20 weeks' gestation.

High fruit intake	N = 58	Case (mtDNA copy number/ml)	N = 117	Control (mtDNA copy number/ml)	OR	(95% CI)
Never	7	2158.15 (1357.11–3119.63)	9	1856.43 (617.47–9759.18)	1.00	1.00–1.00
< 6 times a week	11	3445.15 (1140.94–6661.19)	25	2617.49 (1683.27–4005.63)		
> 5 a day	40	2466.71 (1339.17–4526.35)	83	2829.32 (1868.68–4786.10)		
Never	0		1	6802.61	1.00	1.00–1.00
< 6 times a week	6	2650.57 (1063.15–6136.60)	16	2384.71 (1471.25–5954.11)		
> 5 a day	52	3344.25 (1548.32–7395.89)	108	2856.83 (1980.26–4711.28)		

Table 3(B)
The effect of dietary factors (leafy vegetable intake) on mtDNA in cases compared to controls at 15 and 20 weeks' gestation.

Leafy vegetable intake	N = 58	Case (mtDNA copy number/ml)	N = 117	Control (mtDNA (copy number/ml	OR	(95% CI)
Never	22	2064.62 (1443.17–5873.68)	49	2862.43 (1964.75–5110.42)	1.00	1.00–1.00
< 6 times a week	27	2133.39 (1140.94–4068.91)	42	2547.10 (1644.76–4838.29)		
> 5 a day	9	3119.63 (1677.58–5812.97)	26	2676.89 (1184.76–3950.15)		
Never	1	4198.29 (4198.29–4198.29)	3	1846.20 (1108.22)	1.00	1.00–1.00
< 6 times a week	23	3104.99 (1366.01–7396.91)	46	2596.07 (1636.75–4126.70)		
> 5 a day	34	3315.32 (1518.79–6499.72)	68	3067.12 (2151.85–5347.62)		

Table 3(C)
The effect of dietary factors (Multivitamin intake) on mtDNA in cases compared to controls at 15 and 20 weeks' gestation.

Multivitamin intake	N = 58	Case (mtDNA copy number/ml)	N = 117	Control (mtDNA (copy number/ml	OR	(95% CI)
No	44	2206.55 (1186.20–4709.44)	71	2617.49 (1856.43–4778.04)	1.00	1–1.00
Daily	14	2745.61 (1504.21–6598.51)	39	2850.34 (1626.85–5111.71)		
Less than daily	0		7	3518.35 (1856.43–4315.26)		
No	38	4032.60 (1744.82–7789.96)	57	2777.50 (1769.47–5184.31)	1.00	1.00–1.00
Less than daily	4	1374.57 (332.18–5948.15)	15	3647.68 (2278.63–4711.28)		
Daily	16	2384.71 (1471.25–5954.11)	45	2754.95 (1822.09–4421.01)		

mtDNA (between 15 and 20 weeks' gestation) in cases compared to controls.

Oxidative stress results from an imbalance in the production of ROS and the responsive antioxidant levels. There is a vast amount of evidence for antioxidant decline and elevation of ROS in pre-eclampsia [7,24]. SOD is the first barrier and antioxidant defence against ROS and its activity is increased in the placenta of a normal pregnancy [25], while SOD activity in placental tissue from women with pre-eclampsia is decreased [26]. In our study, we showed lower levels of SOD activity at 15 weeks' gestation in cases compared to controls. This correlates with previous work which showed lower levels of SOD at both 10–14 and 20–24 weeks' gestation respectively in pre-eclampsia [27]. Similarly, the levels of maternal erythrocyte SOD were also lower in the second half of pregnancy in pre-eclampsia when compared with normotensive pregnancies [7].

Mitochondrial DNA is correlated with the number and size of the mitochondria [28], furthermore mtDNA are particularly susceptible to oxidative damage. While the origin of cf-mtDNA is difficult to phenotype, the quantitative assessment of cf-mtDNA may permit the evaluation of mitochondrial dysfunction in pre-eclampsia. There have been a number of studies that suggest mitochondrial abundance may be associated with placental insufficiency and pre-eclampsia [29–32]. Our research previously showed evidence of increased mtDNA at time of disease in women with pre-eclampsia, furthermore, we provided additional evidence of mitochondrial dysfunction by demonstrating increased mitochondrial-specific ROS and reduced oxygen consumption [18]. In this current study, while there was no significant difference in mtDNA copy number at both 15 and 20 weeks' gestation respectively in cases compared with controls, we reported a significant increase in the mean difference in mtDNA copy number between 15 and 20 weeks' gestation in cases compared with controls.

Given the critical role of SOD antioxidants in mediating oxidative damage provoked by exaggerated superoxide generation, the compromised antioxidant defence evident at 15 weeks' gestation in cases in our study group may be partly responsible for increased vulnerability of mtDNA damage as evident by the increase in mean difference in mtDNA between 15 and 20 weeks' gestation. Previous work in retinal endothelial cells overexpressing SOD2 [33] and in SOD2-depleted chondrocytes [34] has established an essential protective role for this enzyme in preventing mtDNA damage. Furthermore, we showed that mtDNA copy number increases as pregnancy progresses in women with pre-eclampsia and we hypothesise that the initial insult to mitochondrial antioxidant function seen early in pregnancy (15 weeks) could be exacerbated later in pregnancy resulting in a more significant increase

in mtDNA copy number in pre-eclampsia as we previously described.

The strength of our data compared to previous work in this area is attributable to the longitudinal examination of mtDNA through gestation, whereas previous studies have focused on mtDNA quantitation in the third trimester of pregnancy. This work correlates with previous studies, where mtDNA copy number was assessed in a case-control study and reported that the odds of pre-eclampsia were positively associated with increased maternal blood mtDNA copy number [31]. These findings strongly suggest that altered mitochondrial function is evident very early in the development of pre-eclampsia. This study was performed in the Irish SCOPE cohort and while we provide evidence of mitochondrial dysfunction in pre-eclampsia, further larger studies in different populations are warranted.

Lifestyle interventions such as healthy diet (fruit and vegetable consumption) and exercise have been examined in mitochondrial diseases [35,36]. In recent years, exercise has been intensively researched in relation to reducing risk of pre-eclampsia [37,38]. The next step in this study was to assess whether lifestyle and nutritional factors had an effect on the amount of mtDNA in controls and cases. However, we showed that exercise had no significant difference on mtDNA copy number during pregnancy in controls or cases. Similarly, a previous study assessed exercise and mtDNA copy number in controls and pre-eclampsia cases and showed no association between exercise and mtDNA copy number [31].

Diet has been suggested to play a potential role in the management of pre-eclampsia [39]. In our study we found no association between fruit and vegetable intake on mtDNA copy number in control and cases. Similarly, Clausen et al, showed no association between pregnancies affected by pre-eclampsia and healthy pregnancies when investigating meat, fish, vegetables and fruit intake [40]. Finally we investigated multivitamin intake and its association with mtDNA copy number in control and cases and found no association. Both Vitamin C and E have been extensively studied as antioxidant therapeutic options in pre-eclampsia [41,42], however the results were largely disappointing. This may have occurred as these exogenous antioxidant vitamins do not penetrate the intracellular source of ROS, the mitochondria, and are sequestered in the cytosol. Therefore, we propose a mitochondrial-targeted antioxidant may represent a more promising clinically effective treatment strategy for pre-eclampsia.

5. Conclusion

In this study, we provide evidence that in early gestation there is a significant reduction in mitochondrial antioxidant SOD activity in women who developed pre-eclampsia. Furthermore, there is a significant increase in ΔmtDNA levels between 15 and 20 weeks' in women who subsequently went on to develop pre-eclampsia. Our findings support a pathogenic role for mitochondrial dysfunction in the pathophysiology of pre-eclampsia. Finally, we found no effect of either lifestyle or dietary factors in mediating mitochondrial dysfunction in this study cohort, highlighting the potential need for the development for mitochondrial targeted antioxidants as potential therapeutic targets to treat pre-eclampsia.

Conflict(s) of interest/disclosure(s)

L.C. Kenny is a minority shareholder in Metabolomic Diagnostics, a campus-based spin-out that has licensed technology concerning the use of metabolomics biomarkers in the prediction of preeclampsia.

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Activation of a TLR9 mediated innate immune response in preeclampsia

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Preeclampsia is a multisystemic disorder leading to the development of a placental ischemic microenvironment with a resultant increase in oxidative stress. There is evidence that mitochondrial dysfunction and the innate immune system both play a role in the pathophysiology of this disease. Mitochondrial DAMPs such as mtDNA bind specific pattern recognition receptors such as Toll-like receptor 9 (TLR9) on the endosomal surface of immune cells, in particular neutrophils, subsequently activating them and triggering an innate response. We hypothesised that the exaggerated innate immune response seen in preeclampsia is provoked by dysfunctional mitochondria. Here we provide evidence that TLR9 activity is significantly increased at time of disease in women with preeclampsia. Furthermore, we show activation of neutrophil markers, Calprotectin, Myeloperoxidase (MPO), and IL-8 are significantly increased at time of disease compared to uncomplicated pregnancies. This research supports a potential role of TLR9 activation of an innate immune response evident in preeclampsia which may possibly be initially triggered by dysfunctional mitochondria.

Preeclampsia is multifactorial disorder of pregnancy that is defined by the onset of hypertension and proteinuria after 20 weeks' gestation. One of the most established characteristics of this disorder is the inability of the trophoblasts to invade the maternal uterine decidual arteries, resulting in poor placentation. Production of placental anti-angiogenic factors such as soluble fms-related tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng) have been extensively researched in pre-eclampsia[1,2]. More recently, the cardiovascular system and its role in the development of preeclampsia is being explored[3]. Other pathological characteristics of preeclampsia include placental and systemic oxidative stress and dysfunction of the maternal vasculature[4,5]. In normal pregnancies there is evidence of a controlled systemic inflammatory response where cytokines promote the infiltration of the spiral arteries by invading trophoblast cells[6]. This controlled inflammatory response becomes dysregulated in preeclampsia resulting in abnormal activation of monocytes, neutrophils and the endothelium causing maternal inflammation[7]. There is overwhelming evidence that oxidative stress plays a key role in the pathophysiology of preeclampsia[8,9]. In turn oxidative stress, as a result of a placental ischemic microenvironment, releases reactive oxygen species into the maternal circulation, which can provoke a systemic inflammatory response[10]. The innate immune system acts as both a protector and effector during pregnancy. The innate system encompasses neutrophils, dendritic cells, natural killer cells and macrophages and these immune responders are activated to protect the mother from pathogens. Toll-like receptors (TLRs) are a family of type I transmembrane pattern recognition receptors (PRRs) that identify invading pathogens or endogenous damage signals and instigate an innate immune response. TLR9 can detect conserved sequences known as pathogen-associated molecular patterns (PAMPs) and also specifically respond to endogenous molecular structures known as damage-associated molecular patterns (DAMPs) via unmethylated CpG dinucleotide motifs as evident on mitochondrial DNA[11]. Activation of endosomal TLR9 involves an intracytoplasmic signalling cascade that leads to the up-regulation of pro-inflammatory transcription factors and subsequent liberation of pro-inflammatory cytokines[12]. There is evidence of increased TLR9 expression in both placental tissue and peripheral blood mononuclear cells (PBMC) from patients with preeclampsia compared with controls[13,14].

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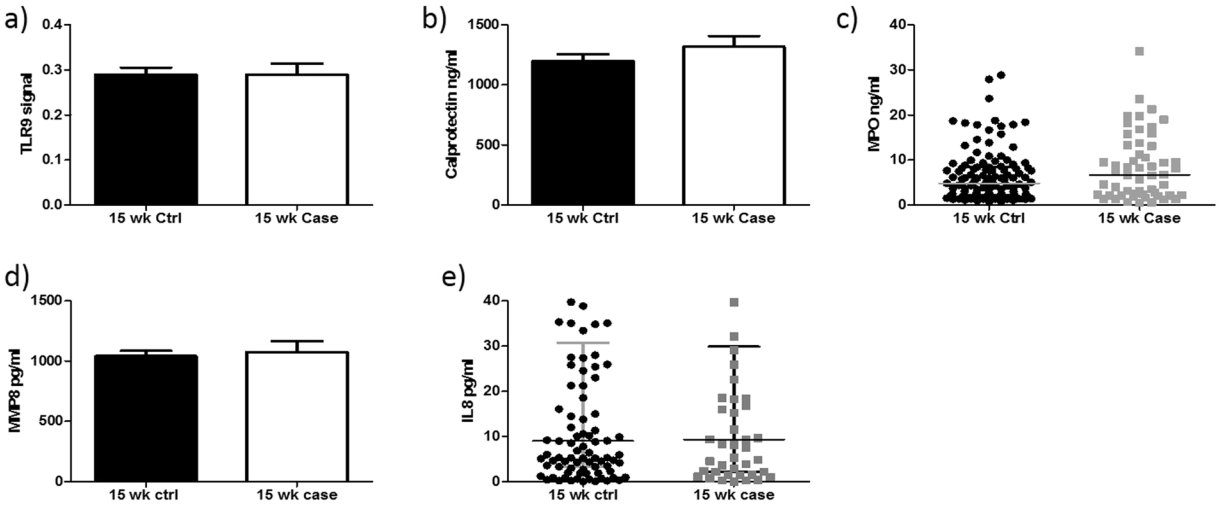

Figure 1. TLR9 activity and downstream markers of neutrophil activation did not alter at 15 weeks' gestation. There was no significant increase in TLR9 activity (**a**) or neutrophil activation markers Calprotectin (**b**), MPO, (**c**) MMP-8 (**d**) and IL-8 (**e**) at 15 weeks' gestation between cases and controls.

Neutrophils are short-lived effector cells of the innate immune system. These immune cells are activated in women during pregnancy and are further stimulated in preeclampsia[15]. Histopathological evidence has shown extensive infiltration of neutrophils in the systemic vasculature of women with preeclampsia compared to controls[16,17]. Neutrophils express a number of TLR's on their surface including TLR9[18]. Mitochondrial DNA (mtDNA) has been shown to activate neutrophils via a TLR9 signalling cascade, which elicits a neutrophil pro-inflammatory phenotype[19–21]. Neutrophil activation results in the secretion of a number of markers including reactive oxygen species (ROS), matrix metalloproteinase-8 (MMP-8), calprotectin, myeloperoxidase (MPO) and the pro-inflammatory cytokine IL-8[22].

Our research has previously provided evidence of a role for mitochondrial dysfunction in the pathophysiology of preeclampsia with an increase in ΔmtDNA evident between 15 and 20 weeks in women with preeclampsia[23]. Interestingly we also showed a significant increase in mtDNA at time of disease (TOD) in women with preeclampsia compared to controls[24]. The aim of this study was to investigate if the increase in mtDNA we previously reported in preeclampsia triggers activation of TLR9 signalling cascade. We also wanted to determine if a subsequent neutrophil pro-inflammatory phenotype was elicited in preeclampsia compared to controls by measuring a number of neutrophil activation markers longitudinally in pregnancy. We hypothesised that dysfunctional mitochondria provokes an exaggerated innate immune response in preeclampsia.

Results

Determination of TLR9 activity and downstream markers of neutrophil activation at 15 weeks' gestation. To determine if TLR9 was activated by circulating mediators present in preeclampsia, HEK-TLR9 cells were treated with 3% plasma. The level of neutrophil activation markers, calprotectin, MPO, MMP8 and IL-8 were also examined. Firstly, there was no significant increase in TLR9 activity at 15 weeks' gestation in cases compared to controls (0.28 ± 0.02 v 0.2 ± 0.02; $P = 0.99$) (Fig. 1a). We subsequently measured a number of well described markers of neutrophil activation in both study groups at 15 weeks' gestation. There was no significant increase in calprotectin (1319 ng/ml ± 87.96, v 1198 ng/ml ± 56.76; $P = 0.23$) (Fig. 1b) or myeloperoxidase (Median [IQR]: 6.72 ng/ml [2.20–13.13] v 4.83 ng/ml [2.11–8.41]; $P = 0.14$) between cases and controls (Fig. 1c). There was also no significant difference in MMP8 (1041.27 pg/ml ± 42.96 v 1075.62 pg/ml ± 88.24; $P = 0.69$) (Fig. 1d) or the pro-inflammatory cytokine IL-8 (median [IQR]: 8.99 [3.32–30.70] v 9.32 [2.18–29.85]; $P = 0.95$) between cases and controls at 15 weeks' gestation (Fig. 1e).

Determination of TLR9 activity and downstream markers of neutrophil activation at 20 weeks' gestation. There was no significant increase in TLR9 activity at 20 weeks' gestation in cases compared to controls (0.29 ± 0.01, v 0.30 ± 0.02; $P = 0.88$) (Fig. 2a). While there was no change in the levels of calprotectin between cases and controls at 20 weeks' gestation (1155 ng/ml ± 68.45 v 1234 ng/ml ± 61.10; $P = 0.42$) (Fig. 2b), there was a significant increase in myeloperoxidase in cases compared to controls (Median [IQR]: 5.02 ng/ml [2.36–9.08] v 7.07 ng/ml [2.74–17.24]; $P = 0.02$) (Fig. 2c). There was no significant change in the levels of MMP8 between cases and controls (1116.73 pg/ml ± 48.24 v 1053.74 pg/ml ± 54.05; $P = 0.41$) (Fig. 2d). There was no significant increase in IL-8 in cases compared to controls at 20 weeks' gestation (Median [IQR]: 7.95 pg/ml [2.52–30.31] v 13.49 pg/ml [3.66–46.66]; $P = 0.14$) (Fig. 2e).

Activation of TLR9 activity provokes a neutrophil-derived pro-inflammatory phenotype at time of disease in preeclampsia. There was a significant increase in TLR9 activity at TOD in preeclampsia cases compared to controls (0.29 ± 0.01 v 0.35 ± 0.01; $P = 0.01$) (Fig. 3a). Subsequently, there was also a significant increase in both calprotectin (1946.55 ng/ml ± 155.08 v 1516.45 ng/ml ± 145.84; $P = 0.04$) (Fig. 3b) and

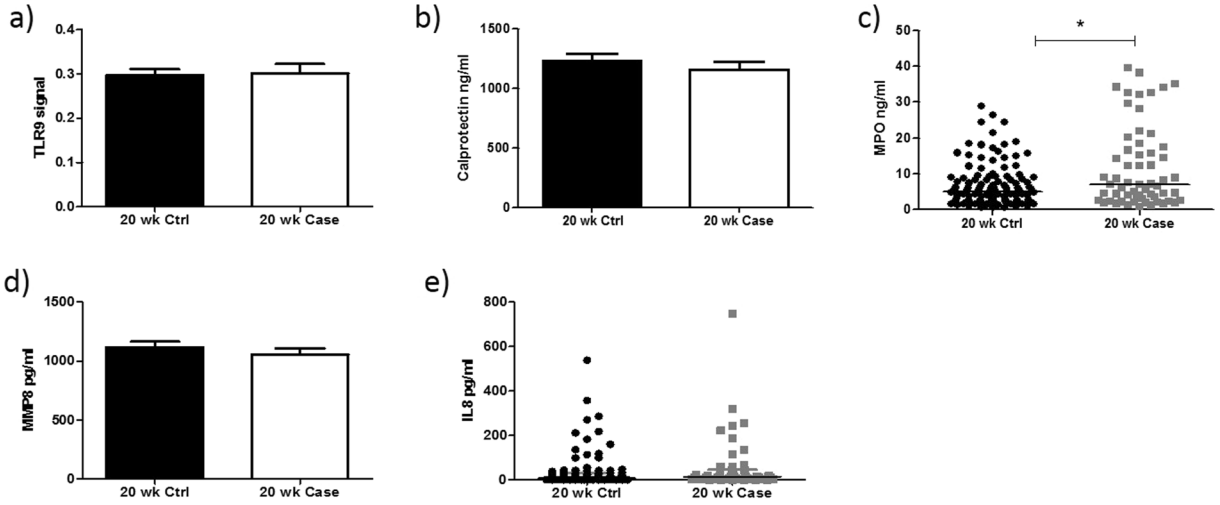

Figure 2. TLR9 activity and downstream markers of neutrophil activation did not alter at 20 weeks' gestation. There was no significant increase in TLR9 activity (**a**) or neutrophil activation markers Calprotectin (**b**), MMP-8 (**d**) and IL-8 (**e**) at 20 weeks' gestation between cases and controls. MPO expression (**c**) is significantly increased in cases compared with controls (P = 0.02).

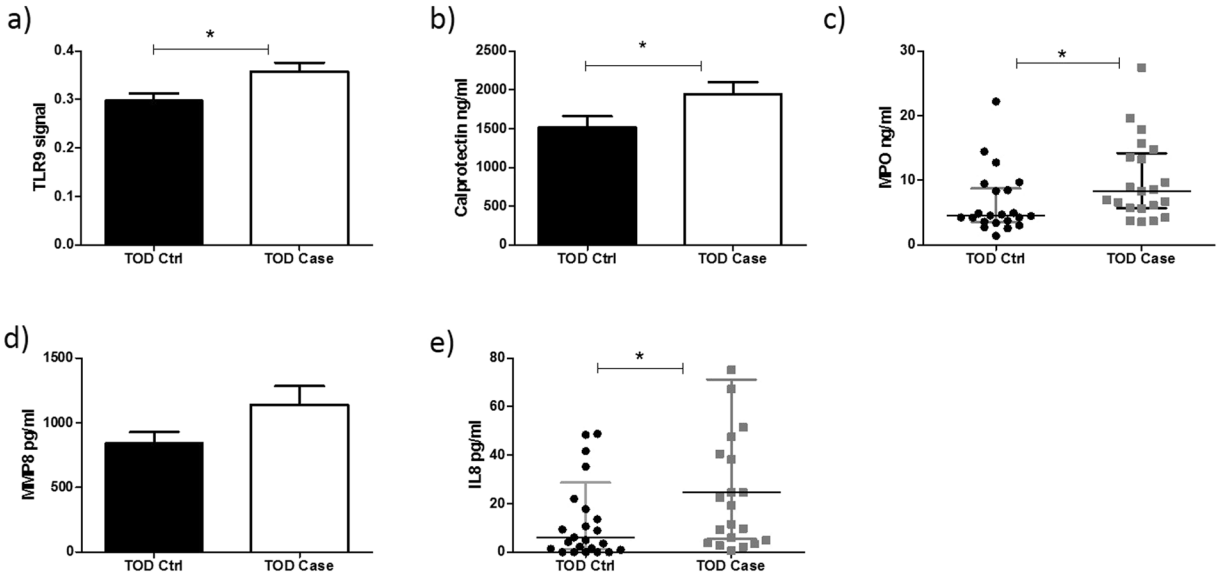

Figure 3. Circulating plasma mediators activate a TRL9-mediated innate immune response in preeclampsia at TOD. TLR-9 activity (**a**), Calprotectin (**b**), MPO (**c**), and IL-8 (**e**) are significantly increased at time of disease in cases compared to controls (P = 0.01). There was no significant increase in MMP-8 at TOD in cases in comparison to controls.

myeloperoxidase (Median [IQR]: 8.33 ng/ml [5.70–14.20] v 4.52 ng/ml [3.53–8.73]; P = 0.01) (Fig. 3c) respectively at TOD in cases compared to controls. There was no significant increase in MMP-8 in cases when compared with controls at time of TOD, (842.92 pg/ml ± 87.07 v 1140.64 pg/ml ± 144.77; P = 0.08) (Fig. 3d). Finally, there was a significant increase in the pro-inflammatory cytokine IL-8 (Median [IQR]: 6.13 pg/ml [1.18–28.68] v 24.63 pg/ml [5.37–71.20]; P = 0.01) at TOD in cases compared to controls (Fig. 3e).

Evidence of elevated innate immune response across gestation in preeclampsia. We investigated if the neutrophil-mediated innate immune response were altered longitudinally in pregnancy. Initially, there was no difference in TLR9 activity across gestation in controls. (Fig. 4a). Similarly, there was no significant change in calprotectin, MPO and IL-8 expression in controls (Fig. 4b,c,e). MMP-8 was significantly decreased in uncomplicated pregnancies (Fig. 4d).

In cases complicated with preeclampsia, both TLR9 and calprotectin were significantly increased across gestation (0.28 ± 0.02, 0.30 ± 0.02, 0.37 ± 0.02; P = 0.01) and (1318.69 ng/ml ± 87.95, 1233.77 ng/ml ± 68.45,

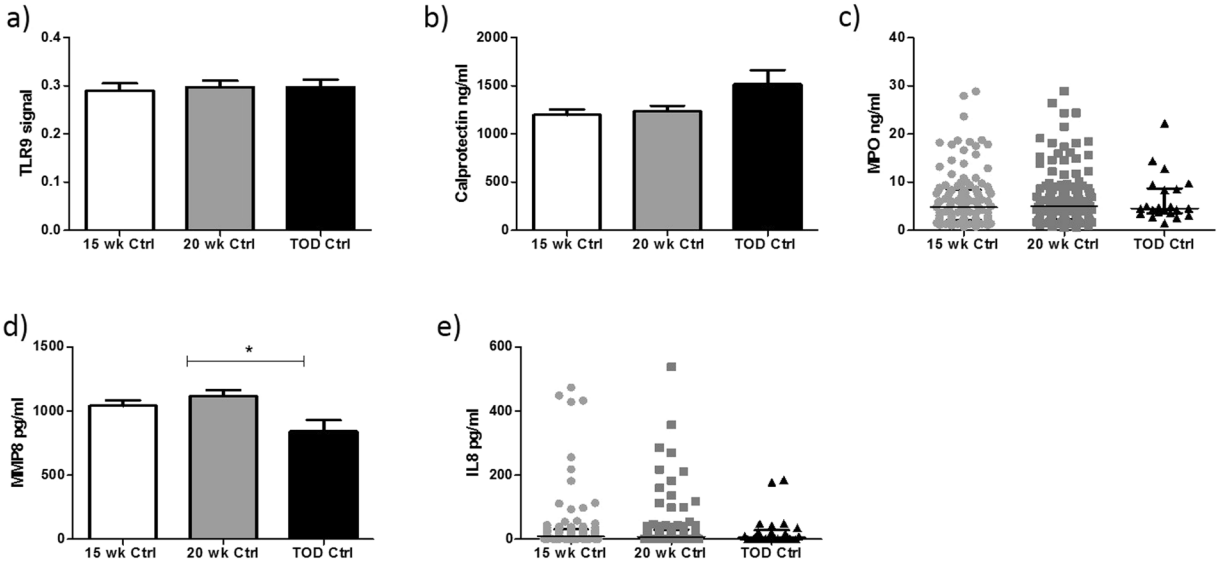

Figure 4. Normal pregnancy did not activate a TLR9 mediated innate immune response. There was no significant increase in TLR9 activity (**a**), Calprotectin (**b**), MPO (**c**) in healthy control pregnancies across gestation. MMP8 (**d**) is significantly reduced at term pregnancies (P = 0.01), while IL-8 (**e**) showed no significant difference across gestation in healthy control pregnancies.

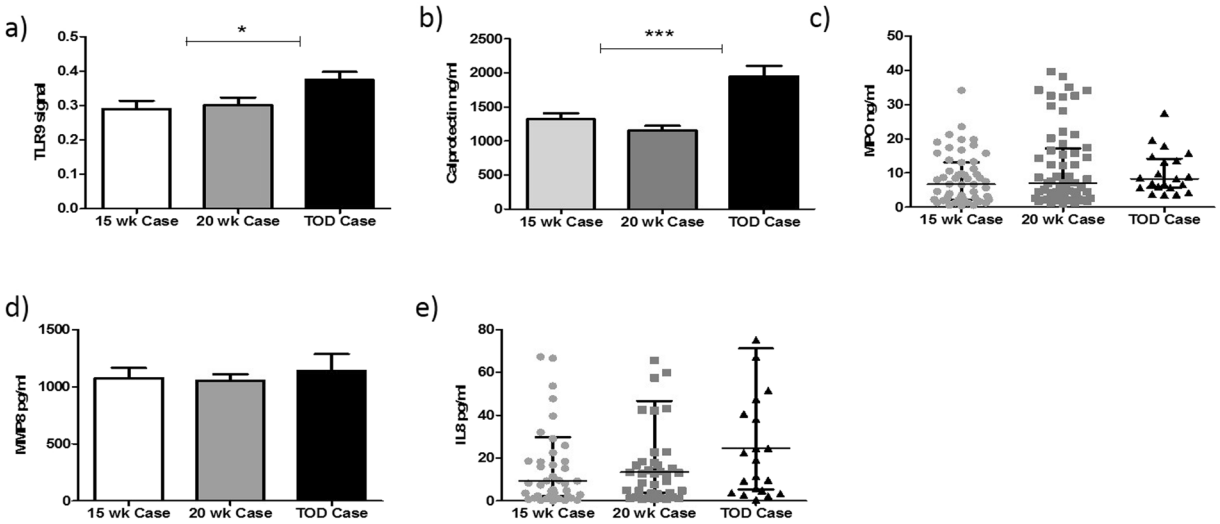

Figure 5. Neutrophil activation markers are increased across pregnancyin preeclampsia. TLR9 activity (**a**), Calprotectin (**b**), are both significantly increased across gestation in cases with preeclampsia (P = 0.01). There was no significant increase in neutrophil activation markers MPO (**c**), MMP8 (**d**) and IL-8 (**e**) across gestation in cases with preeclampsia.

1946.55 ng/ml ± 145.84; P = 0.0001) respectively. (Fig. 5a,b). There was no significant increase in MPO, (6.72 ng/ml [2.20–13.13], 7.09 ng/ml [2.74–17.24], 8.33 ng/ml [5.70–14.20]; P = 0.14), MMP-8 (1075.62 pg/ml ± 88.24, 1053.74 pg/ml ± 54.05, 1140.64 pg/ml ± 87.08; P = 0.82) or IL-8 (9.32 pg/ml [2.18–29.85], 13.49 pg/ml [3.66–46.66], and 24.63 pg/ml [5.37–71.20]; P = 0.13) across gestation in preeclampsia cases (Fig. 5c–e).

Determination of TLR9 activity and downstream markers of neutrophil activation in preterm cases versus term cases. We next explored if there was a difference in the neutrophil pro-inflammatory phenotype across all time-points in women who developed preeclampsia and delivered either before 37 weeks or after 37 weeks' gestation (Table 1). There was no significant difference in TLR9 activity in preterm preeclampsia compared to term preeclampsia across gestation. Similarly, there was no statistical significance at each time-point when comparing expression of calprotectin, MPO and MMP8 in preterm preeclampsia versus term preeclampsia. Interestingly IL-8 expression is initially increased at 15 weeks' gestation in women who developed preterm preeclampsia, but the cytokine levels reduce as pregnancy progresses.

Neutrophil markers	15 weeks (Preterm N = 19)	15 weeks (Term N = 38)	P Value	20 weeks (Preterm N = 19)	20 weeks (Term N = 37)	P Value	TOD (Preterm N = 6)	TOD (Term N = 16)	P Value
TLR9 Signal	0.31 ± 0.05	0.27 ± 0.03	0.66	0.30 ± 0.06	0.29 ± 0.03	0.41	0.37 ± 0.03	0.39 ± 0.04	0.713
Calprotectin (ng/ml)	1043.15 ng/ml ± 126.19	1645.66 ng/ml ± 187.30	0.16	886.99 ± ng/ml 245.48	1167.96 ng/ml ± 77.80	0.57	1878.35 ng/ml ± 424.90	2055.57 ng/ml ± 170.62	0.77
MPO (ng/ml)	4.42 ng/ml [1.77–10.25]	6.58 ng/ml [2.11–9.70]	0.56	4.55 ng/ml [2.48–8.74]	6.15 ng/ml [2.96–21.46]	0.84	8.59 ng/ml [4.72–11.87]	8.33 ng/ml [5.67–14.78]	0.43
MMP8 (pg/ml)	1138.41 pg/ml ± 149.24	989.38 pg/ml ± 113.31	0.25	1097.48 pg/ml ± 108.58	1098.46 pg/ml ± 88.69	0.97	1775.73 pg/ml ± 466.30	1263.75 pg/ml ± 182.92	0.12
IL-8 (pg/ml)	32.09 pg/ml [7.40–192.91]	8.12 pg/ml [2.19–18.25]	0.17	22.92 pg/ml [4.76–188.69]	12.67 pg/ml [2.11–18.23]	0.32	24.63 pg/ml [2.68–47.59]	24.63 pg/ml [9.78–75.14]	0.81

Table 1. Plasma levels of TLR9 activity, neutrophil activation markers and pro-inflammatory cytokine IL-8 in patients with preterm and term preeclampsia. Data is presented as mean ± SEM and median (25–75 percentiles) where applicable.

Discussion

Pregnancy is associated with a maternal systemic inflammatory response; however, this response is exaggerated in preeclampsia. There has been a number of studies which have provided evidence of increased neutrophil activation in women with preeclampsia[16,25,26]. In this present study, we investigated if circulating mediators (including mtDNA)[23], trigger TLR9 activity with the subsequent downstream activation of a neutrophil-mediated innate immune response in women with preeclampsia and healthy controls. We showed TLR9 activity is significantly increased in women with preeclampsia compared to healthy controls at time of disease. There was a corresponding increase in production of downstream neutrophil activation markers, calprotectin, myeloperoxidase, MMP8 and the pro-inflammatory cytokine IL-8 in women with preeclampsia compared to healthy controls at time of disease, indicating that complete activation of neutrophil pro-inflammatory phenotype only became evident late in pregnancy in preeclampsia.

The innate immune response plays a well described role in the pathophysiology of preeclampsia. Mitochondrial DAMPs such as mtDNA bind specific pattern recognition receptors such as TLR9 on the endosomal surface of the immune cells, in particular, neutrophils and activate an innate response. TLR9 receptors are also localised to trophoblasts and the villous stromal vascular endothelium and their expression is increased in preeclampsia[13]. We have shown a significant increase in TLR9 activity using a reporter cell assay incubated with plasma taken at time of disease in preeclampsia cases compared to healthy controls. Furthermore, previous work in our lab using the same study group samples examined mtDNA (as a marker of mitochondrial dysfunction) and reported that mtDNA was also significantly increased at time of disease in preeclampsia cases in the same study cohort[23]. This indicates that the increase in mitochondrial DAMP (mtDNA) at time of disease may activate a TLR9 mediated innate immune response in preeclampsia cases only. Exciting new work by He *et al*. has identified an additional role for TLR9 in preeclampsia where they established that TLR9 supressed angiogenesis in part by increasing sFlt-1 expression in a murine model of preeclampsia[27]. Furthermore, research carried out in spontaneous hypertensive rats, illustrated circulating mtDNA may lead to the activation of the innate immune system through TLR9[21]. Research by Goulopoulou *et al*., has also shown preliminary data indicating that TLR9 activation provokes preeclampsia–like symptoms in pregnant rats[20] emphasising the pathogenic role of TLR9 in preeclampsia.

Calprotectin is a calcium binding protein and is located in the cytosol of neutrophils and is released upon neutrophil activation. In our study, calprotectin was significantly increased at time of disease compared to controls. This is in agreement with recent studies which have reported increase in calprotectin at term in preeclampsia[26]. Akçum *et al*., reported increased circulating calprotectin levels at term in women with preeclampsia, and interestingly found an additional increase in women with severe preeclampsia.

MPO is a lysosomal enzyme mainly produced and released by activated neutrophils. In this study, we showed a significant increase in MPO in cases as early as 20 weeks' gestation when compared with controls and this significant increase was equally evident at time of disease in preeclampsia. Previous studies measuring MPO in preeclampsia have reported conflicting results; some studies have reported no difference in MPO in preeclampsia in samples taken at 24 weeks or later in the third trimester[28,29], whereas Gandley *et al*., reported a 3-fold increase in circulating MPO levels in women with preeclampsia compared to matched healthy controls at 32–38 weeks' gestation[30]. The variation in MPO levels in previous preeclampsia studies may be due to small sample size. In our study we compared 60 cases and 120 matched controls, which to our knowledge is one of the biggest studies carried out in relation to MPO and preeclampsia.

MMP8 is part of the MMP family which consists of 23 zinc and calcium dependent proteases that effect different mechanisms of the extracellular matrix. In recent years, MMPs have become a target of interest in preeclampsia due to its role in vascular function and remodelling[31,32]. Recent research shows strong evidence that MMP8 play an important role in mediating endothelial cell angiogenesis[32]. Furthermore, endothelial dysfunction is a pathogenic characteristic of preeclampsia, dysregulated MMP8 expression may play a crucial role in the disruption of angiogenesis in preeclampsia leading to endothelial dysfunction. In our study, there was an increase in circulating levels of MMP8 expression at TOD in women with preeclampsia cases compared to healthy controls.

Cytokines and chemokines have been extensively studied as markers of inflammation involved in the pathophysiology of preeclampsia. In this study, we showed a significant increase in circulating IL-8 levels at time of disease in cases compared to controls. IL-8 has previously shown to be increased in women with preeclampsia

in the third trimester[33]. A recent study reported a significant increase in IL-8 in early gestation (5–15 weeks') in women who subsequently went on to develop preeclampsia. However, a limitation of their study was the small sample size (n = 9)[34]. Other studies investigating IL-8 throughout pregnancy reported similar results to ours with no significant increase evident in the second trimester[35,36] but a significant increase in IL-8 is evident in the third trimester in women with preeclampsia compared to healthy controls[37].

Preterm preeclampsia occurs before 37 weeks' gestation and is frequently regarded as a slightly different phenotype to preeclampsia occurring at term. Therefore, we investigated whether neutrophil activation triggers an altered innate immune response in preterm preeclampsia compared to term preeclampsia. In this study, there was no statistically significant difference in the concentration of neutrophil activation makers in preterm versus term in our cohort.

When investigating the activation of a TLR9 mediated innate immune response across pregnancy, there was no significant difference in expression of any of the neutrophil markers measured in healthy controls. However, in preeclampsia, significant increases were seen in neutrophil activation markers, TLR9 and calprotectin, illustrating that the innate immune response may be initially triggered earlier in preeclampsia but complete activation is not significantly evident until later in pregnancy. This is in agreement with recent studies of maternal inflammation (cytokines such as IL-6, TNF-α) in preeclampsia, whereby low level inflammation is evident early in pregnancies but is amplified in the third trimester of pregnancy[38–40]. This current study shows that the TLR9 activation of the innate immune system may play a role in the pathophysiology of preeclampsia in late gestation.

Conclusion

Here we provide evidence that circulating plasma mediators may activate a TRL9-mediated innate immune response in preeclampsia. We show that the activity of TLR9, a receptor for mtDNA, is significantly increased at time of disease in preeclampsia. Subsequently we have shown increased production of neutrophil activation markers particularly late in pregnancy in preeclampsia. Finally, we have shown that possible activation of TLR-9 by dysfunctional mitochondria may provoke an exaggerated neutrophil-mediated innate immune response in preeclampsia.

Material and Methods

Study subjects. Subjects were recruited from the Screening for Pregnancy Endpoints (SCOPE) study Ireland which is an international multicentre prospective cohort study of nulliparous singleton pregnancies aimed to develop a screening test to predict adverse pregnancy outcomes including preeclampsia, SGA infants and spontaneous pre-term birth[41,42]. A nested case-control study within SCOPE Ireland was conducted which included all preeclampsia cases in SCOPE Ireland and matched controls with a case-to-control ratio of 1:2. Preeclampsia cases was defined as a systolic blood pressure $\geq$140 mm Hg and/or diastolic blood pressure $\geq$90 mm Hg on at least two occasions 4 hrs apart after 20 weeks' gestation and with proteinuria (24 hour urinary protein $\geq$300 mg or urine dipstick protein $\geq$+2). Randomly selected controls were taken from healthy pregnant women who had uncomplicated pregnancies which were defined as pregnancies not affected by preeclampsia, preterm birth or growth restriction and delivered at >37 weeks. All blood pressure readings were <140 and/or <90 mmHg prior to the onset of labour. These were matched with the cases for maternal age, body mass index (BMI) and gestational age. Both 15 and 20 week samples were taken from the SCOPE study from women who subsequently went onto develop preeclampsia (n = 60) and controls (n = 120). Samples were also taken from a subset of women (n = 25) at the time of disease (TOD) with preeclampsia and matched controls. The 60 women with preeclampsia were composed of 39 women who developed term preeclampsia and 21 preterm preeclampsia cases. The SCOPE study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all the procedures were approved by the Clinical Research Ethics Committee of the Cork Teaching (EMC5(10)05/02/08), and all women provided written informed consent.

Sample collection. Plasma samples were collected in BD Heparin Vacutainer tubes, placed on ice, and centrifuged at 2,400 g for 10 minutes at 4 °C according to a standardised protocol. Plasma samples were stored at −80 °C until analysis.

TLR-9 activity. TLR9 ligand activity was monitored with HEK-blue TLR9 Reporter Cell assay (InvivoGen). All experiments were performed using a cell density of 50,000 cells in a 96 well plate. Cells were initially seeded and left overnight prior to treatment for 24 hrs with 3% plasma taken at 15 and 20 weeks' gestation (cases, n = 60, controls, n = 120) and TOD (cases, n = 25, controls, n = 25). The supernatant was incubated with Quanti-Blue detection medium (InvivoGen) and the activity was read on a Varioskan Flash plate reader (Thermo Scientific) at 630 nm.

Calprotectin analysis. Plasma calprotectin (S100A8/S100A9) concentrations were measured by enzyme-linked immunosorbent assay (ELISA) using human S100A8/S100A9 Quantikine kit. Heparin plasma samples from all time-points were initially diluted a 100-fold in assay buffer and then directly added to a pre-coated plate. The ELISA was performed as per manufacturers' instructions.

Myeloperoxidase, MMP8, IL-8 analysis. Myeloperoxidase (MPO), Matrix metalloproteinases-8 (MMP8) and interleukin-8 (IL-8) concentrations were measured respectively at all time-points by individual ELISA DuoSet kits (R&D SYSTEMS. USA & Canada). ELISA was carried out as per manufacturer's instructions.

Statistical analysis. Analysis was performed using GraphPad Prism. Data were presented using median ($\pm$Interquartile range [IQR]) and comparisons of data between cases and controls were performed using a non-parametric Mann Whitney U test or Wilcoxon signed rank test as appropriate when data was not normally

distributed. Data that was normally distributed were represented as mean (±SEM) and comparisons of data between cases and controls were performed using an unpaired t-test. P values < 0.05 were considered as statistically significant.

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Author Contributions

Conceived and designed experiments: C.Mc.C. and R.W. R.W. performed all experiments. Analysed data: R.W., C.Mc.C. Contributed reagents/materials: C.Mc.C., L.K. R.W., F.Mc.C., C.Mc.C. wrote the manuscript with advice and assistance from L.K.

Additional Information

Competing Interests: L.C. Kenny is a minority shareholder in Metabolomic Diagnostics, a campus-based spin-out that has licensed technology concerning the use of metabolomics biomarkers in the prediction of preeclampsia.

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Title: The nutraceutical antioxidant L-(+)-ergothioneine regulates mitochondrial function in an *in vitro* model of placental oxidative stress

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Abstract:

Introduction: Placental oxidative stress due to abnormal placentation is proposed to play a vital role in the pathophysiology of preeclampsia. There is preliminary evidence implicating mitochondrial dysfunction as a mediator of exaggerated oxidative stress, which is evident in preeclampsia. The aim of this study was to investigate the potential therapeutic properties of L-(+)-ergothioneine, a promising antioxidant, in an *in vitro* model of oxidative stress.

Methods: JEG-3 placental choriocarcinoma cells were pre-treated with 1mM L-(+)-ergothioneine, 20µM Mito-Tempo and 1mM N-acetylcysteine for 2hrs and subsequently treated with 200µM H_2O_2 for 4hrs. Cell viability, mitochondrial-specific superoxide levels, mitochondrial mass and oxygen consumption rate were measured.

Results: Pre-treatment with L-(+)-ergothioneine significantly increased cytoprotection in JEG-3 cells while also significantly reducing mitochondrial-specific superoxide (100% v 48.4% ± 13.8%, n=3; $P \leq 0.01$). Compared with MitoTempo and N-acetylcysteine, L-(+)-ergothioneine had a more pronounced effect across all measured parameters. The orchestrator of mitochondrial function, PGC-1α, as well as UCP-1 were significantly altered in cells pre-treated with L-(+)-ergothioneine and subsequently exposed to oxidative stress. Furthermore, pre-treatment with L-(+)-ergothioneine increased the maximal (uncoupled) oxygen consumption rate of the cells.

Discussion: Here we provide evidence of L-(+)-ergothioneine's potential protective activity and mechanisms in an *in vitro* model of oxidative stress. L-(+)-ergothioneine alleviated the effects of the H_2O_2 oxidative insult by reducing mitochondrial-specific superoxide generation. We provide evidence that ERG mediates its protective effects, at least in part, by regulating markers involved in mild mitochondrial uncoupling while increasing the maximal respiratory rate, enabling cells to be better equipped to respond to oxidative stress.

Keywords: L-(+)-ergothioneine , mitochondrial reactive oxygen species, antioxidant, preeclampsia, oxidative stress

Disclosures: DBK is a named inventor on a patent application involving the biotechnological production of L-(+)-ergothioneine in yeast.

Introduction:

Preeclampsia is characterised as novo hypertension in pregnancy (BP ≥140 mm Hg systolic or ≥90 mm Hg diastolic) at or after 20 weeks' gestation, accompanied by proteinuria and/or evidence of maternal acute kidney injury (AKI), neurological features, haemolysis, liver dysfunction, or fetal growth restriction [1]. Annually, preeclampsia results in over 500,000 fetal and neonatal deaths and more than 70,000 maternal deaths globally [1]. There is substantial evidence that defective placentation in early pregnancy is a vital event in the development of this condition [2]. Furthermore, the ischemic placental microenvironment is initially triggered by a reduction in placental perfusion due to oscillations in oxygen delivery to the placenta and fetus, in turn resulting in exaggerated oxidative stress [3]. Inflammation is also a significant accompaniment [4]. To date, research has shown that abnormally elevated oxidative stress is present in preeclampsia as early as 8-10 weeks' gestation [5].

There are many sources of reactive oxygen species (ROS) within the cell; however, mitochondria are the dominant cellular producer of ROS [6]. There is growing evidence that mitochondrial dysfunction may play a role in the pathophysiology of preeclampsia [7,8] as there are elevated levels of mtDNA in plasma from women with preeclampsia when compared with matched healthy controls [6]. Mitochondrial DNA is generally sequestered inside mitochondria; however after physiological stressors such as trauma, infection, or oxidative stress, mtDNA molecules are detectable in the circulation [9]**.** Furthermore, there is evidence of diminished mitochondrial antioxidant defence in preeclampsia as early as 15 weeks' gestation [8,10].

The placenta requires an extensive quotient of functional mitochondria to meet the elevated metabolic demands of pregnancy. To maintain the appropriate number, function and morphology, there needs to be a balance between fusion and fission [11,12]. Peroxisome

proliferation-activated receptor ɣ co-activator 1α (PGC-1α) is a transcriptional coactivator that regulates numerous auxiliary biological functions associated with mitochondria, including antioxidant defense system, angiogenesis, cellular respiration and oxidative metabolism [13,14]. Furthermore, PGC-1α directs a wide programme of mitochondrial gene expression of mitochondrial-ROS detoxifying enzymes including superoxide dismutases (SOD1, SOD2) and uncoupling protein-1 (UCP-1), which are directly induced by this transcriptional orchestrator [15]. UCP-1 is a mitochondrial transporter present in the inner mitochondrial membrane with a well-known role in mediating adaptive thermogenesis [14]; more recently it has been shown to regulate exaggerated mROS production, in part via mild uncoupling [16].

L-(+)-ergothioneine (ERG) is a dietary, water-soluble amino acid derived from histidine, cysteine and methionine. It is synthesised by various bacteria and fungi and taken up by higher organisms. Many physiological roles have been proposed for ERG including immune regulation, scavenging of free radicals and cation chelation [17]. Furthermore, ERG has been shown to possess cytoprotective and antioxidant properties in both *in vitro* and *in vivo* models [17-25], in addition to being involved in anti-inflammatory responses [26]. ERG crosses the intestinal epithelium using the type 1 organic cation transporter SLC22A4 (previously known as OCTN1) [27]. SLC22A4 was initially considered to be a carnitine transporter; however it transports ERG at a rate almost two orders of magnitude higher [28,29], indicating that the ability to accumulate ERG was selected by evolution. ERG accumulates in conditions of high oxidative stress, indicating that it may be an encouraging agent for diseases involving oxidative stress, such as preeclampsia [30,31]. ERG is a commercially available amino acid, has proven pharmacovigilance for consumption in humans [32], and no adverse effects were seen in pregnant Sprague Dawley rats [33], indicating its potential as a safe treatment for humans, as confirmed by the European Food Standards Agency [34,35].

The aim of this study is to investigate the mechanistic role of ERG in modulating mitochondrial function in an *in vitro* model of placental oxidative stress, and to assess its potential role as a novel therapeutic agent for preeclampsia.

2.0 Materials and Methods:

2.1 Cell culture and treatments.

The human JEG-3 choriocarcinoma placental cell line (ATTC), which are representative of extravillous trophoblasts were grown in Ham's F-12 (Invitrogen) media supplemented with 10% Fetal bovine serum (FBS), 1% L-glutamine and 1% Penicillin Streptomycin (Biosciences). Cells were maintained at 37℃ and 5% CO_2 and were routinely passaged at 80% confluency. All experiments were performed on cells seeded 24-48 hrs in advance and serum starved for 1 hour prior to treatments. Cells were pre-treated initially with increasing concentrations of L-(+)-ergothioneine (1µM–1mM) and Mito-Tempo (1µM-100µM), and N-acetylcysteine (NAC) (1µM-1mM) for 24hrs. For co-treatments, cells were pre-treated with optimal concentrations of 1mM L-(+)-ergothioneine, 20 µM Mito-Tempo and 1µM N-acetylcysteine (NAC) for 2 hrs and 200µM H_2O_2 was then added for either 24hrs, except for mitochondrial superoxide and mass measurements where cells with treated with 200µM H_2O_2 for 4hrs. The oxidative stressor insult (200µM H_2O_2) was chosen based on our previous *in vitro* investigations [36].

2.2 Assessment of Cell Viability using MTT assay

The thiazolyl blue tetrazolium bromide MTT assay assesses cell viability, cells were treated as described in section 2.1 and were incubated at 37℃ with MTT solution for 4 hrs. Following treatment, 10µl of MTT (final concentration 5 mg/ml) was added to each well and

absorbance read at 570 nm, with 630 nm as a reference. Cell viability % = absorbance of each treated cells/absorbance of control- (DMSO-) treated cells x100.

2.3 Isolation of RNA and Real-time PCR analysis

RNA was extracted using the RNeasy mini-kit (Qiagen). Transcripts encoding Peroxisome proliferator-activated receptor-gamma coactivator (PGC-1α), superoxide dismutase (SOD1), SOD2, uncoupling protein-1 (UCP-1) and SLC22A4 were quantified by real-time PCR using the StepONE Plus Detection system. Taqman assay (Applied Biosciences) and Sybr Green primers were used for quantification. The amounts of the target gene was normalised to the geometric mean of internal control gene 18S, and were determined using the comparative $2^{-\Delta\Delta CT}$ method [37].

2.4 Detection of mitochondrial superoxide by fluorescence microscopy

MitoSOX Red fluorescence reagent (Invitrogen) was used to measure intramitochondrial superoxide. This fluorogenic dye is specifically oxidised by superoxide anions in the mitochondria. This oxidation reaction produces a red fluorescent dye once bound to the mitochondrial DNA. Cells were treated as described in section 2.1. Media were then removed and 0.5μM MitoSOX red was added to the cells for 30 mins at 37˚C. Cells were then fixed and permeabilized prior to nuclear staining with DAPI. Mean fluorescence intensity was analysed using Image J software in at least 10 random fields of view and compared to DMSO controls.

2.5 Determination of mitochondrial mass

Mitochondrial mass was measured using the MitoID green fluorescent marker (Enzo Life Sciences) according to the manufacturer's instructions. MitoID is a cell-permeable small

organic probe that is considered to localise to the mitochondria irrespective of any membrane potential they might possess. All experiments were performed at a cell density of 50,000 cells per well in a 12 well plate. Cells were serum starved for 1 hour and subsequently pre-treated with either 1mM L-(+)-ergothioneine, 1mM NAC or 20 μM Mito-Tempo for 2 hrs. Thereafter, cells were treated with 200 μM H_2O_2 for 4 hrs.

2.6 Mitochondrial Oxygen Consumption (OCR)

Mitochondrial bioenergetics were assessed using the Seahorse XF 96 Extracellular Flux Analyser and XF Wave Analysis software version 1.4 and Seahorse XF 96 extracellular flux assay kits (Agilent Technologies, Santa Clara, CA, USA). JEG-3 cells were seeded at 45,000 cells/well on Seahorse XF96 cell culture microplates in DMEM media containing 10% FBS, 1% L-glutamine and 1% Penicillin-Streptomycin. All experiments were performed on cells seeded 24 hrs in advance and serum-starved for 1 hour prior to treatments. Cells were pre-treated with 1mM ERG or vehicle for 6 hrs. All chemicals used to evaluate mitochondrial bioenergetics were from Agilent Technologies (Santa Clara, CA, USA). Following ERG treatment, cell culture media were replaced with non-buffered DMEM-based XF modified media containing 10 mM glucose and 1 mM pyruvate and baseline respiration rates were recorded.

To evaluate mitochondrial function, oligomycin A (1μM), inhibitor of mitochondrial membrane adenosine triphosphate (ATP) synthase, Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP)(0.3μM), an uncoupler of oxidative phosphorylation, and a mixture of antimycin A (1μM) and rotenone (1μM), which are complex I and complex III inhibitors, were used. The instrument was programmed for 3 cycles of drug injection followed by 3 mixing steps and 3 measuring periods (3 min each). The overall experiment time was 110 mins. All oxygen consumption rate (OCR) analyses were carried out

at least three times with a minimum of 15–20 technical replicates for each treatment and normalized to total cellular protein content using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Dublin, Ireland).

2.7 Statistical analyses

Analyses were performed using GraphPad Prism. Data were presented as mean (±SEM), or fold change relative to control. Comparisons of data between *in vitro* groups were performed using one-way ANOVA and Mann Whitney U-Test. For analysing the differences over the course of the Seahorse assay, two-way Repeated Measures ANOVA, followed by Bonferroni post-hoc test was applied.

3.0 Results:

3.1 Determine the preferred concentration of antioxidants on cell viability

To examine the preferred concentration of ERG, JEG-3 cells were treated with a range of increasing concentrations (1μM to 1mM) for 24 hrs. In summary, 1mM ERG was used for subsequent experiments (Figure 1A). Similar cell viability assays were performed with a mitochondrial-targeted antioxidant Mito-Tempo (1μM- 100μM) (Figure 1B) and NAC a non-mitochondrial antioxidant (1μM-1mM) (Figure 1C). In summary, 20μM Mito-Tempo and 1mM of ERG and 1mM NAC were used in subsequent experiments.

3.2 Cytoprotective effects of ERG on H_2O_2 -induced cell death.

The next set of experiments was designed to determine whether ERG had cytoprotective properties on H_2O_2-treated cells. H_2O_2 is commonly used as a cellular stressor representative of oxidative stress in *in vitro* models. 200 μM H_2O_2 was used for subsequent experiments (Figure 1D). Pre-treatment with 1mM ERG significantly rescued H_2O_2 –induced cell death (91% ± 5% v 47 % ± 10.0%, n=3, P= 0.01), when compared with the mitochondrial-targeted antioxidant Mito-Tempo (72% ± 8% v 47% ± 10%, n=3, P=0.11). Furthermore, the ERG-mediated protective effect was similar to that of the non-mitochondrial antioxidant NAC (96% ± 2% v 47% ± 10%, n=3, P= 0.007) (Figure 1E).

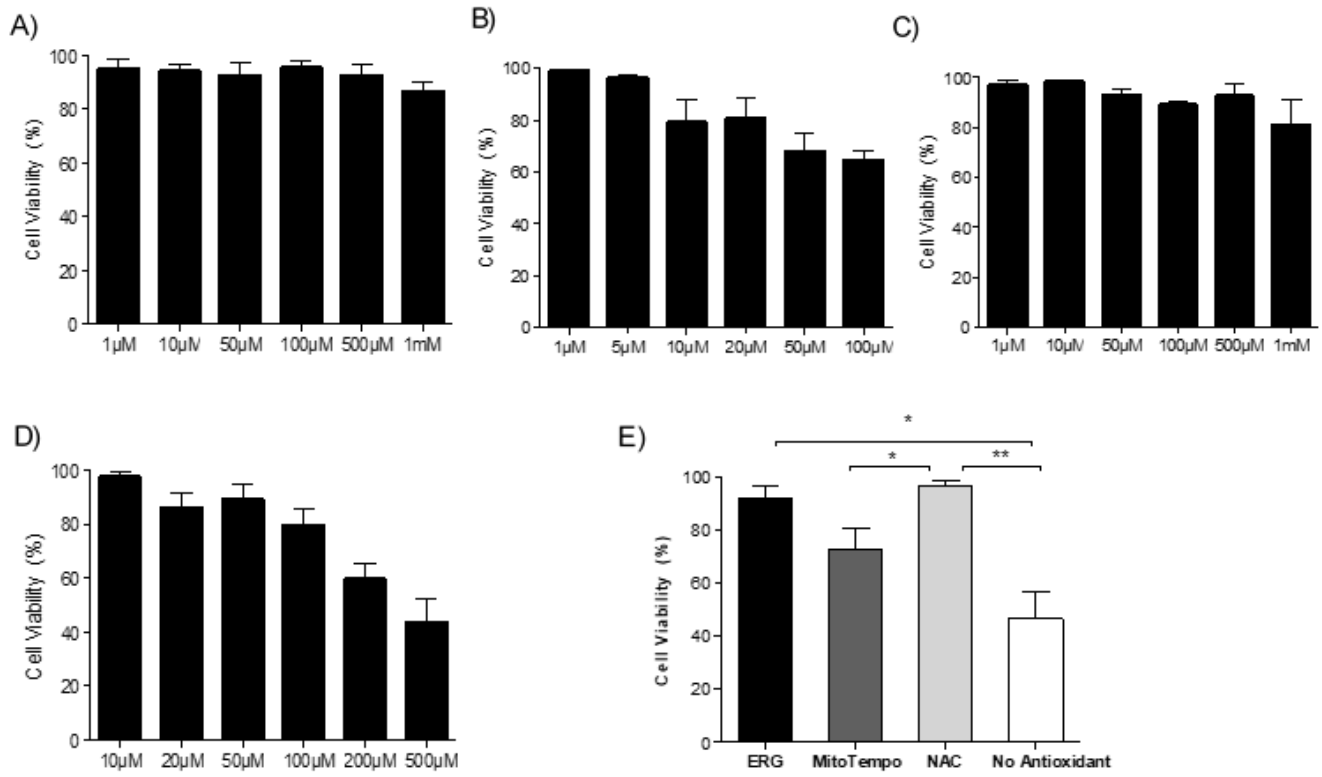

Figure 1: Cytoprotective effects of ERG, MitoTempo and NAC on H_2O_2 –induced-cell death. A-C) The dose-dependent effect of ERG, MitoTempo and NAC on JEG-3 cell viability was assessed using a MTT assay. JEG-3 cells were treated with varying concentrations of ERG, MitoTempo and NAC for 24 hrs. Data are expressed as mean ± SEM. D) Dose dependent effect of H_2O_2 on JEG-3 cell viability was assessed using a MTT assay. JEG-3 cells were treated with varying concentrations of H_2O_2 for 24 hrs. Data are expressed as mean ± SEM. E) ERG is protective of H_2O_2-induced cell death in JEG-3 cells. Cells were pre-treated with 1mM ERG, 20µM MitoTempo and 1mM NAC for 2 hrs and subsequently exposed to 200µM H_2O_2 for 4 hrs, cell viability was assessed by MTT assay. Data are expressed as mean ±SEM. (*P<0.05, **P<0.01; v Control). Data are representatives of 3 independent experiments.

3.3 ERG reduces mitochondrial specific ROS production in H_2O_2 treated cells

Increased production of mitochondrial-specific ROS (mROS) is inherently linked to mitochondrial dysfunction. To examine mitochondrial-specific superoxide production, cells were labelled with MitoSOX Red fluorescent dye and quantified by fluorescent microscopy. Mitochondrial-specific superoxide was significantly reduced in cells pre-treated with ERG (100% v 48% ± 14%, n=3; P<0.01) compared with control cells (Figure 2). Similarly, MitoTempo significantly reduced mitochondrial-specific superoxide production, (100% v 51% ± 12%, n=3; P= 0.01), while NAC didn't significantly reduce mROS production respectively (100% v 68% ± 7%, n=3 P= 0.08).

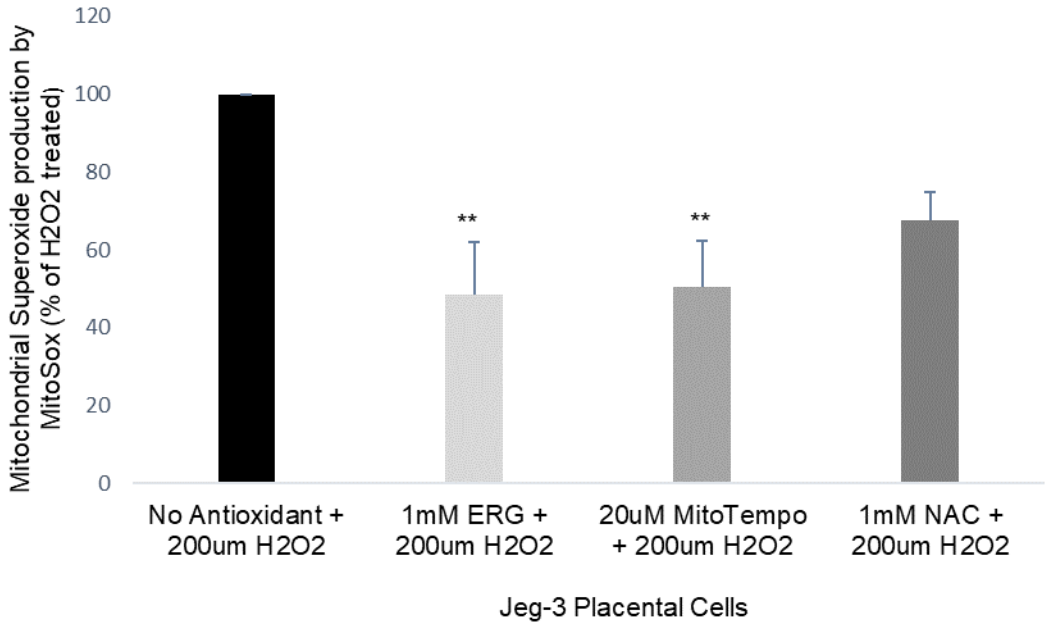

Figure 2: ERG reduced mitochondrial superoxide levels in JEG-3 cells. JEG-3 cells were pre-treated with ERG, MitoTempo and NAC for 2hrs and subsequently treated with H_2O_2 for 4hrs and mitochondrial-specific superoxide was detected using fluorogenic MitoSox Red dye. MitoSox Red generation was quantified using Image J software. Data indicate the mean of 10

independent experiments and are expressed as difference in percentage pixel intensity between study groups ± SEM. *P<0.01.

3.4 ERG did not affect mitochondrial mass in JEG-3 treated cells.

The effect of ERG on mitochondrial mass in JEG-3 cells was measured using fluorescent MitoID Green. MitoID Green accumulates in the mitochondria and becomes fluorescent in a lipid environment, fluorescence was quantified by microscopy. There was no significant change in mitochondrial mass in cells pre-treated with ERG compared to control cells (85% ± 7% v 100%, n=3, P=0.06). Equally, MitoTempo (84% ± 27% v 100%, n=3, P= 0.35) or NAC treated cells didn't significantly alter mitochondrial mass when compared to control cells (81% ± 7% v 100%, n=3; P=0.06) (Figure 3).

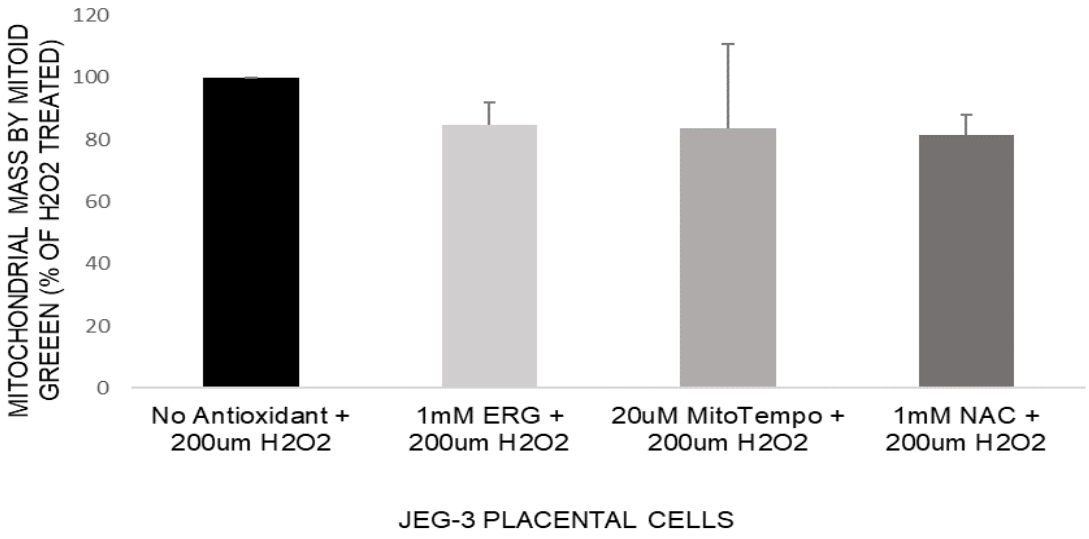

Figure 3: Effect of antioxidants on mitochondrial mass in H_2O_2 treated cells. JEG-3 cells were pre-treated with ERG, MitoTempo and NAC for 2 hrs and subsequently treated with H_2O_2 for 4hrs. Mitochondrial mass was determined using fluorogenic MitoID green reagent. Mitochondrial mass was quantified using Image J software. Data is the mean of 3 independent experiments and are expressed as difference in percentage pixel intensity between group's ± SEM. *P <0.05.

3.5 ERG regulates endogenous UCP-1 expression in placental cells

We assessed the effect of ERG treatment on expression of PGC-1α and its directly regulated mitochondrial target genes SOD1, SOD2 and UCP-1. We also measured SLC22A4 expression in ERG-treated cells. This study showed that ERG treatment did not have a significant effect on the expression of PGC-1α, SOD1, SOD2, SLC22A4 in JEG-3 cells compared with untreated cells. (Figure 4). However, UCP-1 expression was significantly increased in cells treated with ERG (2 fold ± 0.5, n=3, P=0.04) compared to untreated cells.

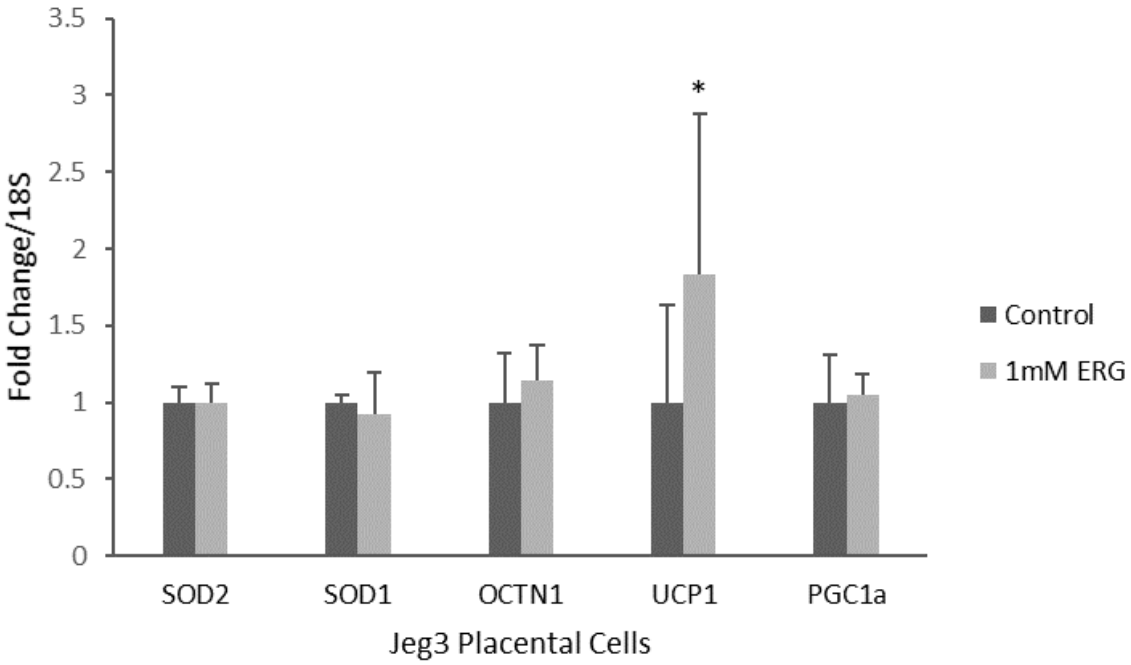

Figure 4: Effects of ERG on endogenous expression of mitochondrial-detoxifying enzymes in untreated JEG-3 cells. JEG-3 cells were treated with 1mM ERG for 24hrs and placental expression of transcripts for mitochondrial-detoxifying enzymes were quantified by real-time PCR. Gene expression of PGC-1α, UCP-1 SOD1, SOD2, and SLC22A4 were

determined in JEG-3 cells. Data from 3 independent experiments are mean fold change compared to untreated JEG-3 cells ± SEM. *P<0.05.

3.6. ERG alters mitochondrial bioenergetics via increasing the maximal (uncoupled) OCR

We characterised the effect of ERG treatment on mitochondrial bioenergetics (Figure 5A) based on the changes in OCR rate in response to modulators of respiration that target components of oxidative phosphorylation. First, ERG treatment did not significantly increase the basal OCR (Figure 5B, 0.72 ± 0.18 pmol/min O_2/µg protein vs. 0.96 ± 0.13 pmol/min O_2/µg protein, p= 0.099) or (as judged by oligomycin-sensitivity) the proportion of basal OCR coupled to ATP synthesis (Figure 5B, 0.56 ± 0.13 pmol/min O_2/µg protein vs. 0.70 ± 0.09, p=0.12) of JEG-3 cells. FCCP administration uncouples respiration from ATP synthesis and removes respiratory control, thereby indicating the maximum OCR the cells could sustain. ERG treatment led to a significantly higher uncoupled OCR rate (Figure 5B, 1.06 ± 0.16 pmol/min O_2/µg protein vs. 0.59 ± 0.15 pmol/min O_2/µg protein, p= 0.02). Finally, there were no endpoint differences detected after the simultaneous administration of rotenone and antimycin A (Figure 5B, 0.46 ± 0.13 pmol/min O_2/µg protein vs. 0.47 ± 0.08 pmol/min O_2/µg protein, p= 0.45).

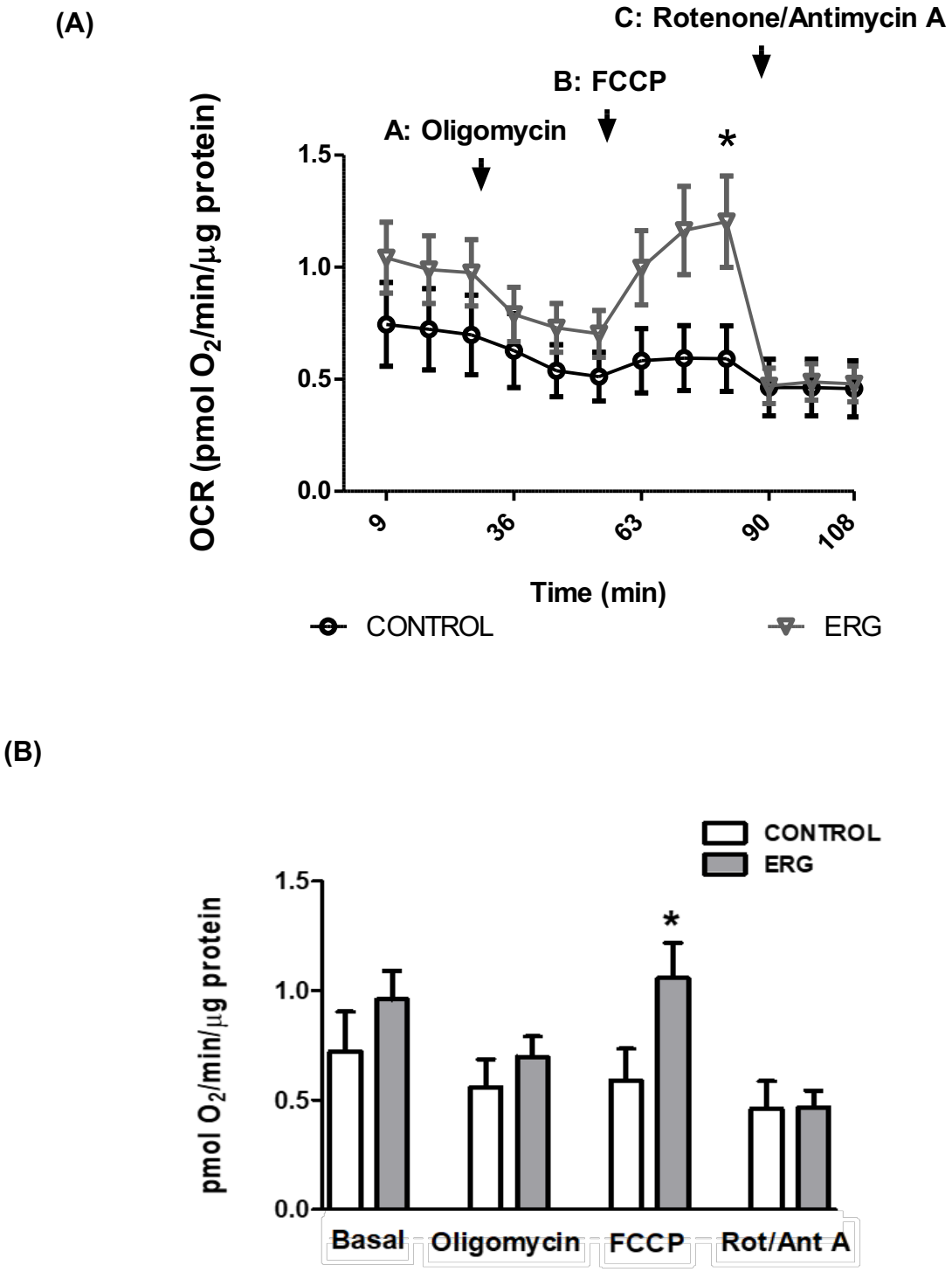

Figure 5. ERG modifies the maximal uncoupling OCR of JEG-3 cells. (A) Bioenergetic profiles of JEG-3 cells treated with 1mM ERG and control for 6hrs. (B) Oxygen consumption rates (OCR), and maximal respiration (OCR after FCCP administration). Data are

representative of 3 independent experiments and expressed as mean ±SEM, (A) Two-way ANOVA, *p<0.05, ***p<0.001 vs Control (B) Mann-Whitney U-test, *p<0.05 vs Control.

3.7 ERG alters the profile of mitochondrial superoxide detoxifying enzymes in response to oxidative stress

ERG significantly increased the expression of PGC-1α (1.8-fold ± 0.3, n=3, P= 0.03) compared to control cells (Figure 6). Additionally, Mito-Tempo (1.5 fold ± 0.2, n=3, P=0.04) and NAC (1.4 fold ± 0.1, n=3, P=0.002) significantly increased PCG-1α expression, albeit with a reduced effect. UCP-1 is known to mediate mROS production in part by mild uncoupling therefore, we wanted to investigate the effect of ERG treatment on UCP-1 expression. ERG significantly increased the expression of UCP-1 (1.6 fold ± 0.3, n=3, P=0.03) compared with controls (Figure 6). Mito-Tempo and NAC did not alter UCP-1 expression. The expression of SOD1, SOD2 or SLC22A4 did not change in response to any pre-treatment strategy.

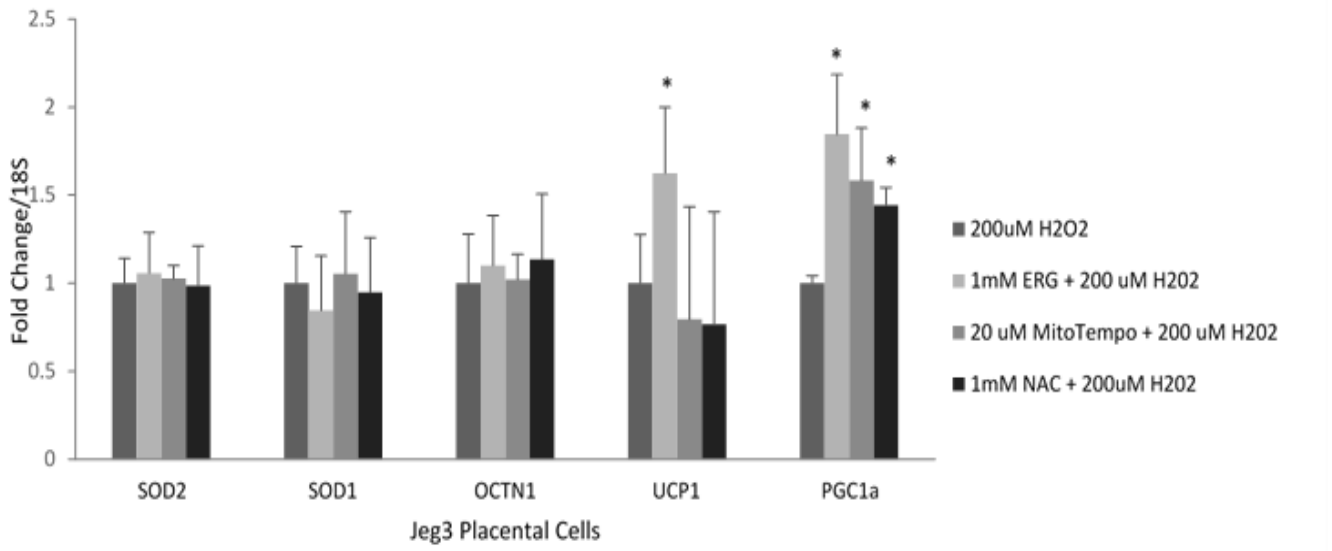

Figure 6: Effect of ERG, MitoTempo and NAC on placental expression of mitochondrial-detoxifying enzymes in H_2O_2 treated cells: JEG-3 cells were pre-treated with ERG, MitoTempo and NAC for 2 hrs prior to exposure to H_2O_2 for 4 hrs and gene expression of markers of mitochondrial-detoxifying enzymes were quantified by real-time PCR. The amounts of amplified products were expressed relative to geometric mean of 18S internal controls. Data are mean fold change compared to controls ± SEM. *P<0.01. Data are representative of 3 independent experiments.

Discussion

It is well known that oxidative stress is a prime mediator in the pathophysiology of preeclampsia, and more recently there has been a specific focus on the role of mitochondrial dysfunction in regulating oxidative stress in preeclampsia [36,38]. However, a number of antioxidants have been notably unsuccessful as treatment strategies for preeclampsia [39,40]. This may be due to the fact that they remain sequestered in the cytoplasm and fail to reach the mitochondria, therefore restricting their capacity to alter the oxidative damage seen in preeclampsia, or because in the presence of free iron they are actually pro-oxidant [6]. The overall aim of this study was to explore the mechanism of action of a novel nutraceutical antioxidant, L-(+)-ergothioneine, in regulating mitochondrial function in an *in vitro* model of placental oxidative stress, and subsequently elucidate its role as a potential novel therapeutic target for preeclampsia.

Initially, we wished to investigate the cytoprotective effects of ERG on H_2O_2-induced oxidative damage in JEG-3 cells. We observed that 1mM ERG significantly preserved cell viability in response to the oxidative stress. Similarly, Paul et al, showed ERG exerted cytoprotective effects on HeLa cells following H_2O_2 treatment. Furthermore, these authors indicated that depletion of SLC22A4 markedly augmented sensitivity to pyrogallol-induced cell damage and death, reinforcing the physiologic antioxidant role of ERG [41]. ERG has previously been used for the treatment of oxidative stress in human brain microvascular endothelial cells (HBMECs) where Li et al., showed ERG reduced cell death as well as oxidative stress [42]. MitoTempo is an extensively studied mitochondrial-targeted antioxidant [36]; therefore, we wanted to compare the efficacy of ERG in protecting mitochondrial function in comparison to the established MitoTempo. We demonstrated that ERG had increased cytoprotective properties when compared with MitoTempo.

In the present study, we showed that ERG significantly reduced mitochondrial-specific ROS production in JEG-3 cells. Similarly, MitoTempo significantly reduced mROS production but with less potency than ERG. We have previously shown that MitoTempo also reduced mROS production in HUVEC cells incubated with plasma from women with preeclampsia [36]. In comparison, ERG was significantly more effective than was the non-mitochondrial antioxidant, NAC in reducing mROS generation, highlighting that ERG may combat placental oxidative stress evident in preeclampsia by regulating mitochondrial reactive oxygen species production.

Mitochondrial biogenesis is involved in the control of cell metabolism, signal transduction and regulation of mROS production. Mitochondrial mass reflects the equilibrium between rates of biogenesis and degradation [43]. We measured mitochondrial mass and found no significant difference in JEG-3 cells pre-treated with ERG which would suggest that the dampening of mROS upon ERG treatment is not directly related to a disruption in mitochondrial mass. We previously reported that mitochondrial-targeted antioxidant MitoTempo reduced mROS generation without altering mitochondrial mass in preeclampsia plasma treated HUVEC [36], a similar effect was also evident in placental cells upon MitoTempo treatment.

PGC-1α is coinduced with numerous ROS-detoxifying enzymes upon exposure of cells to an oxidative stressor and is often required for the activation of many these ROS-detoxifying enzymes, including SOD1, SOD2 and UCP-1 [15]. Under non-pathological circumstances, the production of mROS is strictly regulated by many antioxidant systems in order to sustain redox-signaling homeostasis. We subsequently investigated the effect of ERG on both endogenous and H_2O_2-induced expression of PGC-1α and its target genes UCP-1 and SOD1 and SOD2 antioxidants in JEG-3 cells. We found no significant effect of ERG on SOD antioxidants in either physiological state. Similarly, MitoTempo or NAC had no significant

effect on the expression of either antioxidant. We have previously shown that MitoTempo-mediated reduction of mROS in HUVEC cells also didn't directly alter the expression of SOD antioxidants.

However, we identified a significant increase in both endogenous and H_2O_2-induced expression of both PGC-1α and its downstream target UCP-1, in cells treated with ERG. PGC-1α has previously been shown to be significantly reduced in placental tissue in women with preeclampsia [44], additionally, there was significant evidence of dysregulation of mitochondrial biogenesis in these placental tissues. UCP-1 is directly regulated by PGC-1α [15] and has recently been shown to regulate mROS production, in addition to its recognised role in adaptive thermogenesis (45, 46). Mild uncoupling due to increased UCP-1 expression may participate in an antioxidant defense mechanism to dampen exaggerated mROS production.

Proton leakage back to the mitochondrial matrix from the intermembrane space induced by UCP-1, alters the apparent degree of membrane energisation and also decreases mROS production [46]. In support of this, FCCP-induced OCR was significantly increased following ERG treatment with a resultant increase in maximal respiratory capacity. The reduction in mROS as a result of ERG treatment may be related to this alteration in mitochondrial bioenergetics, by priming the placental mitochondria to be better equipped to combat oxidative insult. While both MitoTempo and NAC had a marginal effect on PGC-1α expression, there was no additional effect on UCP-1 expression suggesting that ERG treatment may recruit alternative transcriptional machinery which amplify the UCP-1 response.

Here we provide evidence that the nutraceutical antioxidant ERG has significant cytoprotective properties in response to oxidative stress. ERG significantly reduced the

generation of mROS without altering mitochondrial mass or antioxidant expression. This study presents novel data that ERG may be mediating its effects via increasing UCP-1 expression which induces mild coupling and while also increasing respiratory capacity, thereby positively modifying the cells capacity to respond to oxidative insult. Finally, our study provides evidence that directly targeting mitochondrial superoxide scavenging with ERG could be a therapeutic candidate for the treatment of preeclampsia.

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Title: L-(+)-ergothioneine significantly improves the clinical characteristics of preeclampsia in the reduced uterine perfusion pressure rat model

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Title: L-(+)-ergothioneine significantly improves the clinical characteristics of preeclampsia in the reduced uterine perfusion pressure rat model

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Short Title: L-ergothioneine as a treatment for Preeclampsia

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Abstract

Preeclampsia is a multifactorial hypertensive disorder of pregnancy founded on abnormal placentation, and the resultant placental ischemic microenvironment is thought to play a crucial role in its pathophysiology. Placental ischemia due to fluctuations in the delivery of oxygen results in oxidative stress, and recent evidence suggests that mitochondrial dysfunction may be a prime mediator. However, large clinical trials of therapeutic antioxidants such as vitamins C and E for the treatment of preeclampsia have been disappointing. L-(+)-ergothioneine (ERG), an unusual amino acid betaine derived from histidine, has important cytoprotective and antioxidant properties under conditions of high oxidative stress. In this study, we investigated the potential therapeutic effects of administration of ERG in the reduced uterine perfusion pressure (RUPP) rat model of preeclampsia.

L-ergothioneine (25mg/kg/day) was administered to rats on gestational day 11. On gestational day 14 RUPP surgery was performed and on gestational day 19, blood pressure (mean arterial pressure) and fetal growth were measured. Production of mitochondrial-specific H_2O_2 was analysed *in vivo* in kidney samples. ERG ameliorated the hypertension and significantly increased pup weight in RUPP rats. ERG also significantly decreased circulating levels of anti-angiogenic sFlt-1 in RUPP rats. Mitochondrial-specific H_2O_2 was also significantly decreased in kidney tissue in RUPP rats treated with ERG. These data support the potential use of ERG for the treatment of preeclampsia.

Introduction

Preeclampsia is a multisystemic disorder of pregnancy which results in high blood pressure accompanied by and/or proteinuria, acute kidney injury (AKI) and/or liver dysfunction fetal growth restriction (FGR) after 20 weeks' gestation (1) It affects more than eight million pregnancies worldwide annually, and is the leading cause of maternal death (2). Despite extensive research, the exact pathophysiological mechanisms underlying this syndrome remain poorly elucidated. Nonetheless, defective placentation is strongly considered to be a critical event in the pathology of the disorder (2). Failure to remodel spiral arteries results in high-pressure blood-flow-mediated placental damage and intermittent fluctuations in oxygen delivery, which exposes the placenta to oxidative stress (3). The resultant placental ischaemic microenvironment is inherently linked to increased production and secretion of deleterious soluble mediators that provoke extensive maternal inflammation and endothelial dysfunction (4). Several reports have observed higher levels of markers of oxidative stress (including F_2-isoprostanes, nitrotyrosine and 4-hydroxynonenal staining) in placental tissue from preeclamptic pregnancies compared with those from uncomplicated pregnancies (5-8).

While there are a number of different cellular sources of reactive oxygen species (ROS), mitochondria are the major cellular producers (9). Furthermore, in terms of preeclampsia, there is growing evidence incriminating mitochondrial dysfunction in its underlying pathophysiology (10). Initial studies showed increased mitochondrial lipid peroxidation and enhanced susceptibility to oxidative damage in placental tissue of pregnancies complicated by preeclampsia (11). More recently, work has confirmed this association with strong evidence of perturbation of mitochondrial

function in the metabolite profile of plasma samples taken at 15 week's gestation from patients who subsequently developed preeclampsia (12).

While there is significant evidence for the pathogenic role of oxidative stress in the development of preeclampsia, clinical trials of antioxidant interventions were disappointing and not clinically effective in treating the disorder. One very plausible explanation is that these antioxidants missed the intracellular location of ROS production, namely the mitochondria; hence they have failed to alleviate the pathological oxidative damage. Another is that molecules such as L-ascorbate can actually be pro-oxidant in the presence of free iron (9). L-ergothioneine (ERG) is an unusual thio-histidine betaine amino acid and is a naturally occurring anti-oxidant discovered over a century ago in the rye ergot (13-16). The predominant role of ERG, via a variety of mechanisms, is to serve as an antioxidant and cellular protectant against various kinds of reactive oxygen species (17). Additionally, there has been some circumstantial evidence that ERG could target mitochondria (18) and hence could dampen exaggerated mitochondrial-specific ROS in response to oxidative stress.

Therefore, we aimed to investigate the role of ERG as a potential therapeutic target for preeclampsia using the reduced uterine perfusion pressure (RUPP) model in pregnant rats. The placental ischaemic RUPP model has numerous features of preeclampsia that are clinically evident in women and has been used as a preclinical model for the investigation of novel therapeutic targets for the treatment of preeclampsia (19, 20). Moreover, we additionally wanted to examine if ERG ameliorated the clinical characteristics of preeclampsia in part via regulation of mitochondrial-specific ROS production. To this end, we used a novel ratiometric mass spectrometry probe MitoB that specifically accumulates in mitochondria and

generates a MitoP phenol product upon reaction with H_2O_2 which can be subsequently be analysed *ex vivo* by mass spectrometry.

Materials and Methods

Animals:

Sprague Dawley-timed pregnant rats were supplied and maintained by the University College Cork Biological Services Unit. Animals were maintained at a temperature of 21˚C, with a 12-hour light/dark cycle and free access to food and tap water. All the procedures were performed in accordance with National Guidelines and the European Directive 2010/63/EU, under an authorization issued by the Health Products Regulatory Authority Ireland and approved by the Animal Ethics Committee of University College Cork (AE19130/P037).

RUPP procedure

The RUPP procedure is a well-established surgical model for studying the link between placental ischemia and hypertension in the pregnant rat and has been previously described in detail (19, 21). In brief, on gestational day (GD) 14, under isoflurane anaesthesia, RUPP reduction in blood flow to the uteroplacental unit was achieved by placing a silver clip (0.2 mm ID) on the abdominal aorta (one clip) above the iliac bifurcation. Two further clips (0.1 mm ID) were carefully placed around the left and right ovarian arteries. Sham surgery was performed as controls, which involved abdominal incision but didn't involve insertion of any clips on either abdominal aorta or ovarian arteries. On GD18, a chronic indwelling catheter was

inserted into the carotid artery and on GD19 mean arterial blood pressure (MABP) was recorded in conscious animals.

L-ergothioneine *in vivo* experimental protocol

Four experimental groups were used to investigate the effect of administration of ERG in the RUPP rat model of pre-eclampsia. Pregnant rats were divided into Sham (n=8), Sham+ERG (n=8) or RUPP (n=8), and RUPP+ERG (n=8). ERG was administered at (25mg/kg/day) in their drinking water on GD11 until the end of the experiment on GD19. The dose for ERG (25mg/kg/day) were selected based on previously published rodent studies using this antioxidant (22, 23). L-ergothioneine was provided by Tetrahedron (Paris, France; www.tetrahedron.fr).

Plasma Collection

Blood collected from EDTA vacutainers were centrifuged at 2000 x g and 2400 x g for 10 minutes at 4˚C, plasma was removed and stored at -80˚C for further analysis.

Urine analysis

On GD18, each rat was singularly housed in a metabolic cage and urine collected overnight. All samples were stored immediately following collection at -80^{o}C. Microalbumin:creatinine ratios were calculated following measurement of albumin using an immuno-turbidimetric test for the quantitative determination of albumin in an OLYMPUS AU5832 analyser® and urine creatinine using a kinetic colour test (Jaffé method) (24). Similarly, protein: creatinine ratios were calculated following measurement of protein by adding benzethonium chloride which resulted in the formation of a fine suspension, which was then quantified turbidimetrically at 525 nm using an OLYMPUS analyser®.

Measurement of anti-angiogenic protein sFlt-1 by ELISA

Circulating sFlt-1 concentration in plasma samples from all experimental groups were quantified by enzyme-linked immunosorbent assay (ELISA) using a Quantikine sFlt-1 immunoassay (R&D Systems) as per manufacturers' instructions.

Isolation of RNA and Real-time PCR analysis

RNA was extracted from placental tissue using the Trizol method. SLC22A4 (ERG transporter), superoxide dismutase 1 (SOD1), SOD2, and uncoupling protein-1 (UCP-1), PCG-1α and Nrf2 gene expression was quantified by Real-time PCR using StepONE Plus Detection system. Taqman assays (Applied Biosciences) and Sybr Green primers were used for quantification. The amounts of the target genes were normalised to the geometric mean of internal control gene 18S, and were determined using the comparative $2^{-\Delta\Delta CT}$ method.

Isometric Myography

In all groups, third order mesenteric arteries were dissected and mounted on a 4-channel wire myograph (Model 610 mol/L Danish Myo Technology) containing oxygenated (95% O_2 and 5% CO_2) physiological salt solution (PSS) at 37˚C. Vessels were normalized to achieve a transmural pressure of 100mmHg using the DMT Normalization software. Isometric tension was recorded and displayed using Powerlab Chart Software (AD Instruments). The viability of the smooth muscle was examined by the addition of a 123 mM KCl solution. After PSS washes, concentration responses were carried out with thromboxane mimetic U46619 (9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$; 10^{-9} to 10^{-5} M) ,and either

Bradykinin (BK; 10^{-8} to 10^{-5} M), Acetylcholine (ACh; 10^{-8} to 10^{-5} M), or Sodium Nitroprusside (SNP; 10^{-8} to 10^{-5} M) respectively (25, 26).

Estimation of mitochondrial H_2O_2 in the RUPP model *in vivo*

Mitochondrial hydrogen peroxide was measured *in vivo* using the Cayman Chemical Hydrogen Peroxide Ratiometric MaxSpec kit based on the MitoB mass spectrometric probe method described previously (27). Briefly, 75 nmol MitoB in 50µl saline was administered by tail-vein injection to rats in the 4 experimental groups on GD19, 4 hours prior to end of the experiment. At the end of the procedure, kidney tissues were dissected out, snap frozen and stored at −80 °C. For mitochondrial H_2O_2 analysis, kidney tissues were homogenised, spiked with deuterated internal standards and MitoB and its product MitoP were extracted using acetonitrile/formic acid. MitoB and MitoP present in kidney tissue were measured using UPLC Xevo TQD mass spectrometer (Waters) and the amounts of MitoP and MitoB in each sample were determined relative to a standard curve. The MitoP/MitoB ratios for each sample were then calculated.

Statistical analysis

All data are expressed as mean ± SEM, or fold change relative to control. Analysis was performed using GraphPad Prism and student *t* test was applied when comparisons were made between 2 groups.

Results

L-ergothioneine ameliorated RUPP-induced hypertension

Mean arterial blood pressure (MABP) was significantly increased in the RUPP group compared with sham group (129 ± 3 mmHg vs 117 ± 7 mmHg, P = 0.05; N=8, Figure 1A). Furthermore, administration of ERG significantly reduced MABP in the RUPP rats (129 ± 3 mmHg v 115 ± 4 mmHg), P = 0.01; N=8, Figure 1A). MABP in sham rats treated with ERG was not significantly different from RUPP rats treated with ERG, indicating the beneficial effect of ERG antioxidant in reducing blood pressure occurs in response to placental ischemia. There was no significant difference in microalbumin:creatinine (MCR) ratio in the RUPP group compared to Sham group, nor was there a significant difference in the MCR ratio in the RUPP rats treated with ERG (Figure 1B).

L-ergothioneine improves fetal weight

Fetal birth weight was significantly decreased in the RUPP group compared to the Sham group (1.8 ± 0.04g v 2 ± 0.03g; P= 0.0004; Figure 2A). Administration of ERG significantly rescued fetal growth restriction in the RUPP rats (2 ± 0.03 g v 1.8 ± 0.04g, P= 0.0006; Figure 2A). Placental weights were significantly reduced in RUPP group compared to Sham group (0.4 ± 0.01g v 0.5 ± 0.01g; P= 0.002, Figure 2B). The administration of ERG had no effect on placental weight in the RUPP rats (0.4 ± 0.01g v 0.4 ± 0.01g) (Figure 2B). A significant decrease in pup number was observed in the RUPP group compared to Sham group (11 ± 1 v 14 ± 1 P= 0.02, Figure 3C). There was no significant difference in pup number in RUPP rats treated with ERG relative to RUPP rats (13 ± 2 v 11 ± 1). Pup weight was not significantly different between Sham or RUPP rats treated with ERG, indicating the beneficial

effect of ERG in rescuing fetal weight during placental ischemia. There was no significant difference in the crown to rump length (CRL) of pups in any of the studied groups (Figure S1. Supplemental Material). Finally, maternal weight did not differ between any of the studied groups. (Figure S2. Supplemental Material).

L-ergothioneine reduces circulating sFlt-1 levels

There was a significant increase in the circulating soluble anti-angiogenic mediator sFlt-1 in the RUPP group compared to the sham group (1995 ± 97 pg/ml v 1185± 349 pg/ml, P= 0.04) (Figure 3). Administration of ERG significantly decreased circulating sFlt-1 levels in RUPP rats (1367 ± 245 pg/ml, P= 0.04; Figure 3).

Effect of L-ergothioneine on vasorelaxation in the RUPP model

Mesenteric arteries from the RUPP group displayed impaired vasorelaxation in response to bradykinin (BK) when compared to the sham group (R_{max}: 28 ± 7% versus 52 ± 9%; P= 0.01, log EC50: -6.6 ± 0.2 versus -6.8 ± 0.3 mol/L; P=0.7, Figure 4A). However, no significant differences were seen in the vascular response to the endothelial independent vasodilator sodium nitroprusside (SNP) (R_{max}: 78 ± 5% versus 80 ± 5%; P= 0.36, log EC50: -7.1 ± 0.2 versus -7.2 ± 0.4 mol/L, P=0.9, Figure S3.A) or Acetylcholine (ACh) (R_{max}: 83 ± 8% versus 81 ± 6%; P= 0.1, log EC50: -7.1 ± 0.2 versus -7 ± 0.1mol/L, P= 0.6 Figure S.3B). Treatment with ERG had no significant effect on the vasorelaxant responses of mesenteric vessels in response to BK (R_{max}: 23 ± 5% versus 28 ± 7%; P= 0.9, log EC50:-6.7 ± 0.2 versus -6.8 ± 0.2 mol/L, P=0.7, Figure 4B), or SNP (R_{max}: 78 ± 6% versus 74 ± 4%; P= 0.8, log EC50: -7.1 ± 0.2 versus -7.2 ± 0.2, P=0.9, Figure 4.SA and B) in RUPP rats.

L-ergothioneine alters placental expression of markers of mitochondrial ROS-detoxifying enzymes

Placental expression of mitochondrial orchestrator proliferator activated receptor γ co-activator 1-α (PGC-1α) (1 ± 0.4 fold vs 0.4± 0.5 fold, n=8, P= 0.01, Figure 5A) and mitochondrial-ROS detoxifying enzymes including uncoupling protein 1 (UCP-1) (1 ± 0.5 fold vs 0.2 ± 0.5 fold, P= 0.04) were significantly decreased in the RUPP rats. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a transcriptional regulator of the mitochondrial antioxidant defence system and coactivated by PGC-1α was also significantly reduced in the RUPP rats (1 ± 0.2 fold vs 0.4 ± 0.4 fold, n=8, P= 0.01, Figure 5A). Placental expression of SOD1 antioxidant was significantly increased (1 ± 0.3 fold vs 1.4 ± 0.3 fold, n=8, P= 0.02; Figure 5B), while mitochondrial SOD2 antioxidant was significantly reduced (1 ± 0.3 fold vs 0.6 ± 0.3 fold, n=8, P= 0.01, Figure 5B) in RUPP rats compared with sham group.

ERG treatment significantly increased placental expression of PGC-1α (1.7 ± 0.3 fold vs 1 ± 0.5 fold, n=8, P= 0.02), UCP-1 (4.9 ± 0.6 fold vs 1 ± 0.5 fold, n=8, P= 0.001) and Nrf2 (1.6 ± 0.3 fold vs 1 ± 0.4 fold, n=8, P= 0.02, Figure 5A) in RUPP rats. ERG treatment significantly increased placental expression of SOD2 in RUPP rats (1.5 ± 0.3 fold vs 1 ± 0.3 fold, n=8, P= 0.02, Figure 5B). There was no significant difference in placental expression of the ERG transporter OCTN1 in any of the experimental groups studied (Figure 5B).

L-ergothioneine reduces mitochondria-specific H_2O_2 production in the kidney

MitoP/MitoB ratio was increased in kidney tissue of RUPP rats compared with the sham group (0.029 ± 0.001 v 0.024 ± 0.006; n=3, P=0.3, Figure 6), indicating for the first time that mitochondrial-specific H_2O_2 is increased *in vivo* as a result of placental ischaemia. Furthermore, pre-treatment with ERG significantly reduced the MitoP/MitoB ratio in kidney tissue in RUPP rats (0.022 ± 0.003 v 0.029 ± 0.001; n=3, P=0.05, Figure 6), establishing that L-ergothioneine reduces mitochondrial-specific H_2O_2 production *in vivo.*

Discussion

Despite a significant amount of evidence for the pathological role of oxidative stress in the development of preeclampsia, clinical data from two major antioxidant vitamin trials have been negative (28, 29). One possible reason for these negative findings may be due to the fact these interventions missed the primary intracellular producer of ROS, namely the mitochondria; or because in the presence of free or poorly liganded iron substances such as ascorbate are actually pro-oxidant (9). In the present study, L-ergothioneine, an amino acid with potent antioxidant properties, attenuated hypertension and rescued fetal growth restriction in the preclinical RUPP rat model of preeclampsia, which closely mimics many aspects of preeclampsia during human pregnancy. Additionally, this study also reported the novel finding of reduced mitochondrial H_2O_2 levels *in vivo* following L-ergothioneine administration. This work has highlighted that L-ergothioneine acts as a potent antioxidant, that ameliorates a number of phenotypic features of preeclampsia in a preclinical model of disease and mediated in part by the reduction of mitochondrial-specific ROS. This work consequently proposes the potential of ERG as a viable therapeutic for the prevention of preeclampsia.

L-ergothioneine is a water-soluble amino acid that is derived entirely from dietary sources. It has garnered much attention recently as a potential therapeutic intervention (13, 30-33), partly due to its preferential accumulation within tissues undergoing significant oxidative stress. A recent study investigating the reproductive safety profile of ERG in pregnant Sprague Dawley rats, established that ERG was well tolerated and with no adverse effects on a number of parameters (number of mating days, gestation length, pup viability index or litter parameters) (34). Furthermore, ERG treatment of diabetic rats significantly improved embryo

formation and quality (31). Collectively these data suggest ERG treatment may be safe to use in pregnancy and may prevent embryo malformations mediated by oxidative stress early in pregnancy. Consequently, we decided to administer ERG at an early point in pregnancy (GD11) in the preclinical RUPP model of preeclampsia.

The therapeutic effects of ERG in response to induced tissue damage in models of ischemia-reperfusion injury in the liver (35) and intestine (22) have previously been reported by the dampening of markers of oxidative stress and inflammation. Our novel data using ERG reported a reduction in hypertension and an improvement in fetal weight in response to placental ischemia in the preclinical RUPP model of preeclampsia, in part due to regulation of mitochondrial-specific ROS. Recent evidence identified a prominent role for mitochondrial-specific ROS (mROS) in modulating hypertension (36)**.** Using two *in vivo* murine models of hypertension (Ang II-induced and DOCA salt), this group established that using a mitochondria-targeted antioxidant (Mito-Tempo) alleviated endothelial dysfunction, reduced vascular mitochondrial superoxide and subsequent hypertension (36). Furthermore, similar to our work, Vaka et al showed in the preclinical RUPP model that placental ischemia dysregulated mitochondrial function with elevated mROS and identified that treatment with mitochondrial targeted antioxidants attenuated hypertension with improvement in fetal outcomes in treated RUPP rats (37).

Deleterious circulating mediators including sFlt-1 are secreted in response to placental ischemia and have devastating consequences on the maternal vasculature. Furthermore, we previously detected increased levels of mitochondrial-specific superoxide production in HUVEC's incubated with plasma from women with preeclampsia compared with matched controls and non-pregnant controls (38). Additionally, Zsengeller et al. established an inverse correlation between placental

sFlt-1 and mitochondrial complex IV, suggesting that sFlt-1 may be harmful to mitochondria in preeclampsia (30). In our study, ERG treatment reduced the circulating levels of sFlt-1 in RUPP rats, indicating that ERG may preserve mitochondrial function in part by reducing the availability of sFlt-1 to induce mitochondrial damage. The reduction in sFlt-1 as a result of ERG treatment did not result in a subsequent reduction in maternal vascular dysfunction in the RUPP rats.

The capacity to accurately measure the concentration of reactive oxygen species, in particular mitochondrial-specific ROS *in vivo*, has proved to be extremely challenging yet is essential in understanding their physiological roles in certain diseases. The detection of mitochondrial-specific ROS such as superoxide using fluorescent probes can be inferred, with certain caveats, by determining the changes in fluorescence, or ex-vivo tissue measurements of ETC complexes but these methods can be less selective and sensitive. One potential alternative is to use exogenous ratiometric probes (MitoB) injected into the animal models, that readily accumulate in mitochondria of tissues due to its possession of a triphenylphosphonium cation and which reacts with H_2O_2 *in vivo* to produce a diagnostic exomarker (MitoP) that can be accurately quantified by mass spectrometry (39).

In this study, we have shown for the first time that this method of measuring mitochondrial H_2O_2 production *in vivo* is applicable in a rat model, without any adverse effects on reproductive safety. Furthermore, we reported that mitochondrial H_2O_2 was increased in kidney tissue in the RUPP group compared with the sham group. This is in agreement with recent work by Vaka et al, who showed an increase in mROS in kidney tissue in the RUPP group by examining ETC activity and cellular respiration *ex vivo* using isolated mitochondria from kidney tissues (37).

More importantly, treatment with ERG resulted in a significant reduction in mitochondrial-specific H_2O_2 production in kidney tissue in RUPP rats, indicating that ERG may be mediating its therapeutic effects in part by directly reducing mROS production in the preclinical model of preeclampsia. Further evidence to support our theory is provided by the fact that placental expression of markers of mitochondrial-ROS detoxifying enzymes and their transcriptional regulators were significantly improved in ERG-treated RUPP rats, in contrast to their reduced expression in RUPP rats.

At present, only one study has investigated the pharmacokinetics of ERG administration in human subjects (30). ERG was rapidly absorbed and retained within the tissue/plasma with relatively low urinary excretion (<4% of administered dose). This work was further extended by Tang et al. who established that ERG and its metabolites are widely distributed in various tissues in male mice administered ERG over a range of days. This group also established that ERG was highly retained in the body and suggested this could be as a result of possible reabsorption by the kidneys (40).

This study provides evidence of the therapeutic potential of ERG in a preclinical model of preeclampsia. ERG improved both hypertension and fetal weight in the RUPP rat model of preeclampsia. Furthermore, ERG treatment significantly altered mitochondrial function in both the kidney and placental tissue which may in part be responsible for the beneficial effects on phenotypic features of preeclampsia in the RUPP model. Given its favourable safety profile, its long half-life and resistance to auto-oxidation and multiple mechanisms of action, not least its ability to regulate mitochondrial function, further studies are needed to explicitly define the protective mechanisms of ERG in treating preeclampsia in humans.

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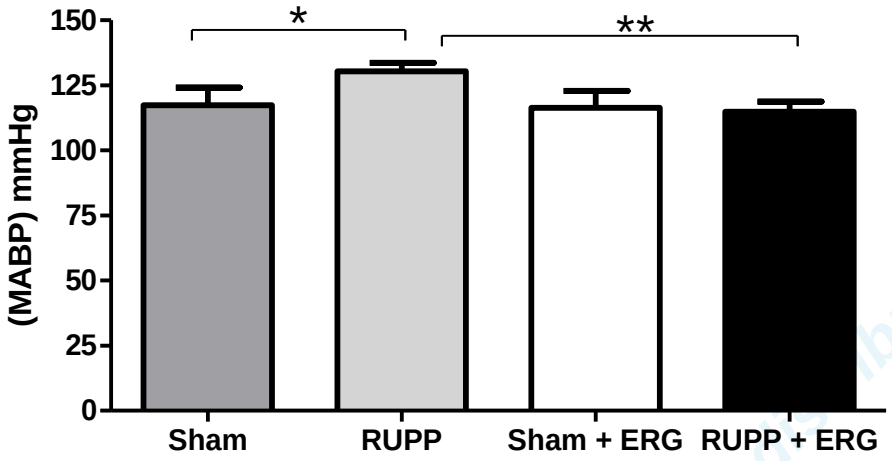

Figure 1A: L-ergothioneine attenuates blood pressure in reduced uterine perfusion pressure (RUPP) rats: a) RUPP rats (n=8) showed significantly elevated mean arterial blood pressure (MABP) compared with sham group (n=8). L-ergothioneine (25mg/kg/day) reduced MABP in RUPP rats (n=8). Data is presented as mean ± SEM, *P<0.05, **P<0.01.

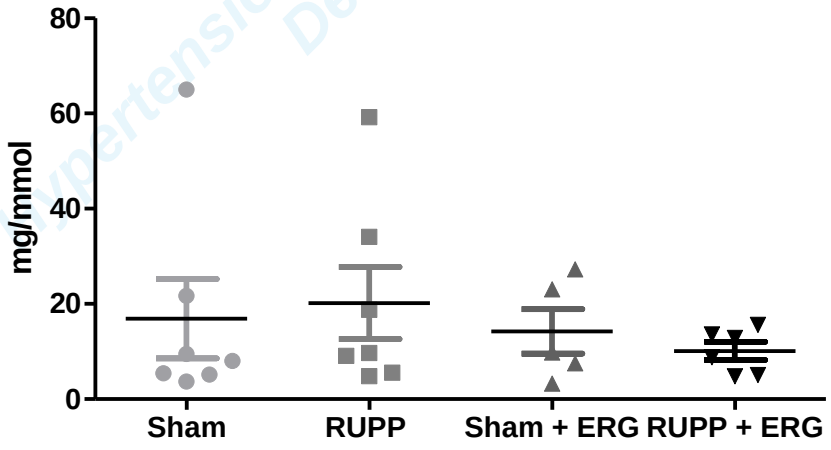

Figure 1B: L-ergothioneine did not alter microalbumin:creatinine ratio in RUPP rats: Administration of L-ergothioneine (25mg/kg/day) did not significantly affect microalbumin:creatinine ratio in RUPP rats.

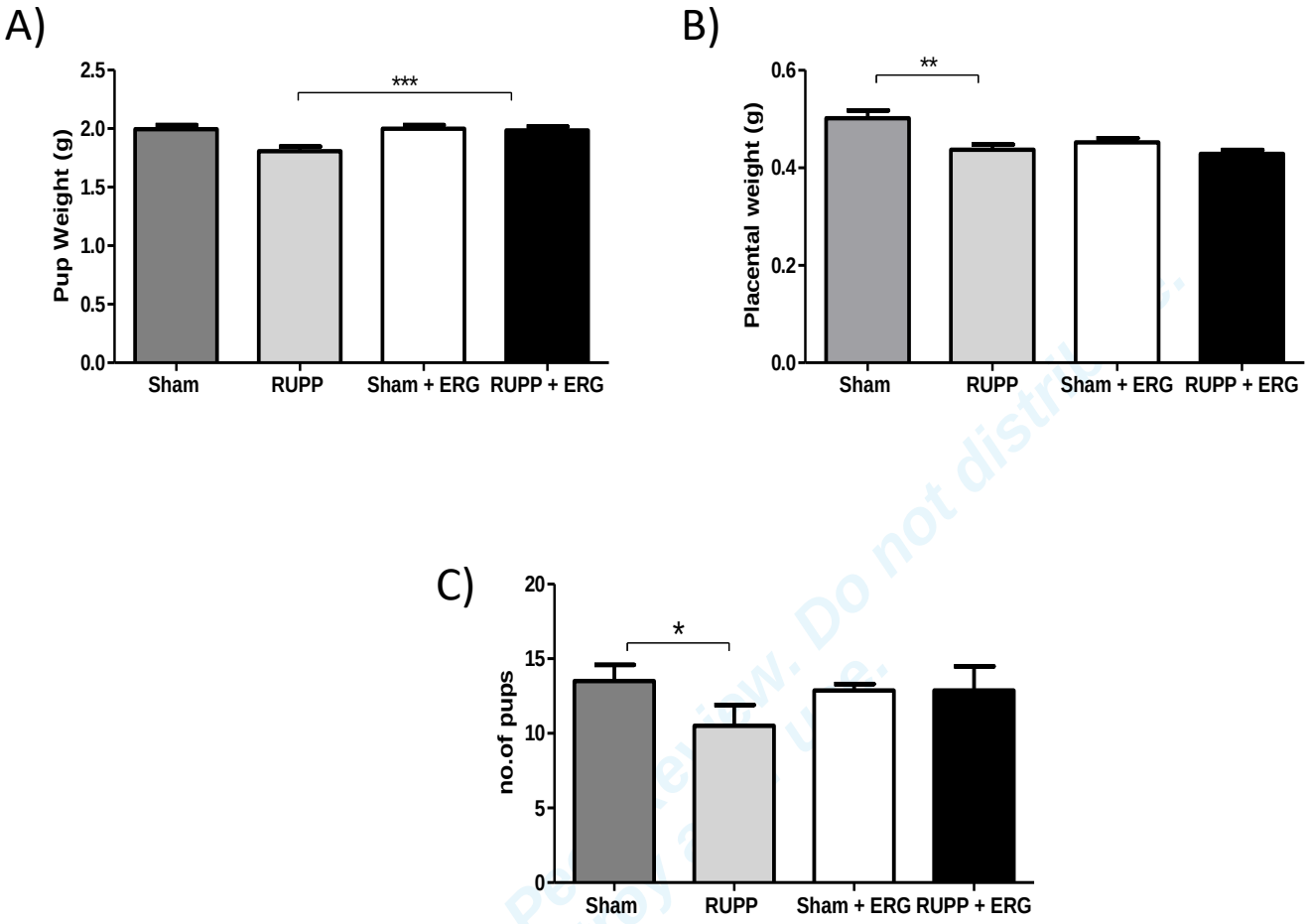

Figure 2: L-ergothioneine improves fetal outcomes in RUPP rats: RUPP rats (n=8) show reduced A) pup weight and B) placental weight and C) litter size compared with sham group (n=8). Administration of L-ergothioneine (25mg/kg/day) improved fetal outcome in RUPP rats. Data are presented as mean ± SEM, *P<0.05, **P<0.01, ***P<0.001.

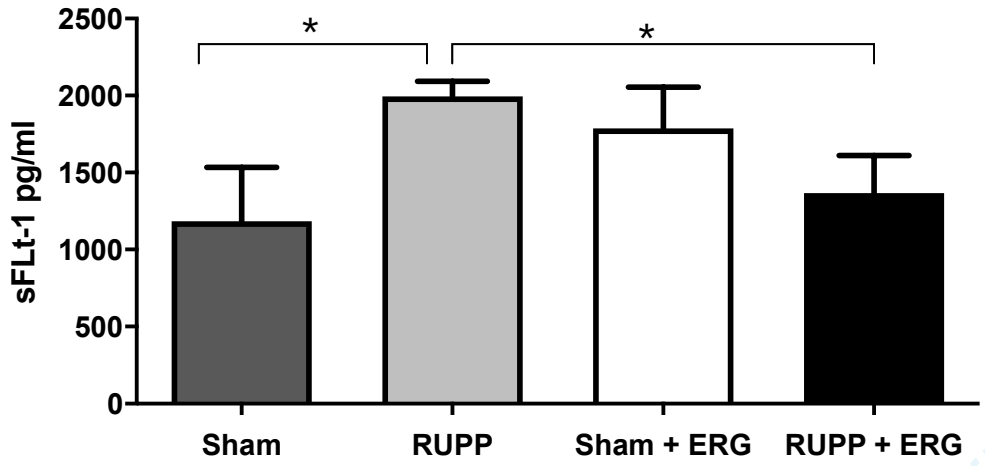

Figure 3: L-ergothioneine reduces circulating anti-angiogenic factor sFlt-1 in RUPP rats: Circulating levels of sFlt-1 were significantly increased in RUPP rats (n=8) compared with sham controls (n=8). Administration of L-ergothioneine significantly reduced circulating sFlt-1 levels in RUPP rats. Data is presented as mean ± SEM, *P<0.05.

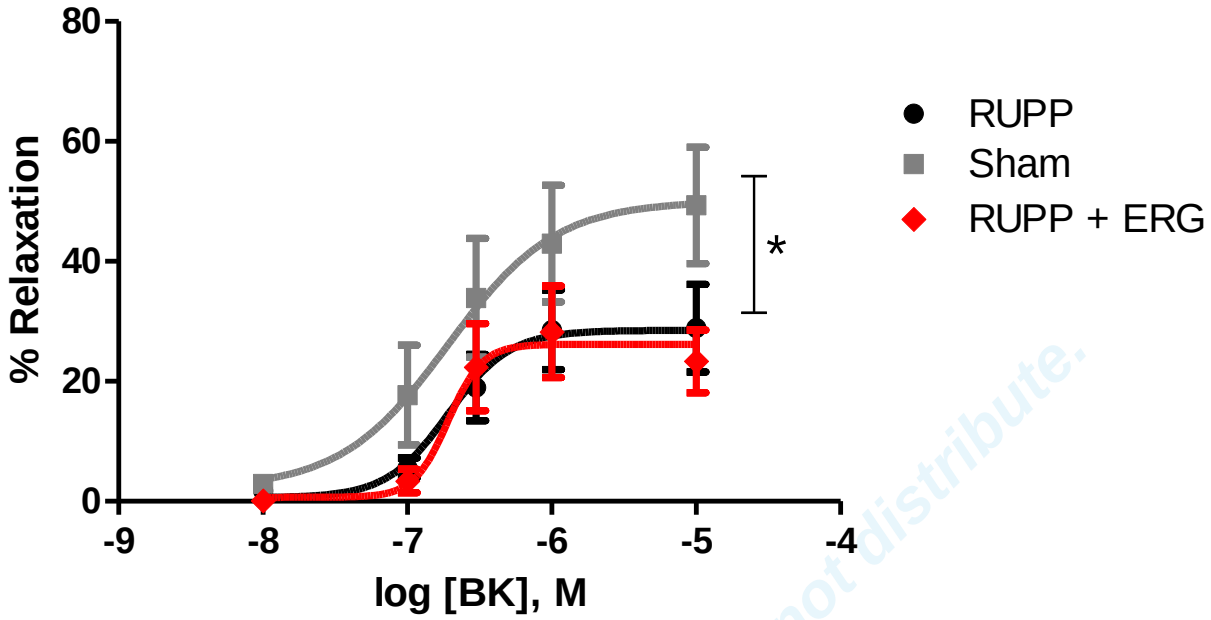

Figure 4: Impaired vasorelaxtion in mesenteric arteries in RUPP rats: RUPP rats (n=8) showed significantly impaired vasorelaxation in response to bradykinin compared to the sham group (n=8). Administration of L-ergothioneine did not attenuate the impaired vasorelaxation to bradykinin. Relaxation is calculated as a percentage of the maximum contraction and expressed as mean ± SEM. *P<0.05.

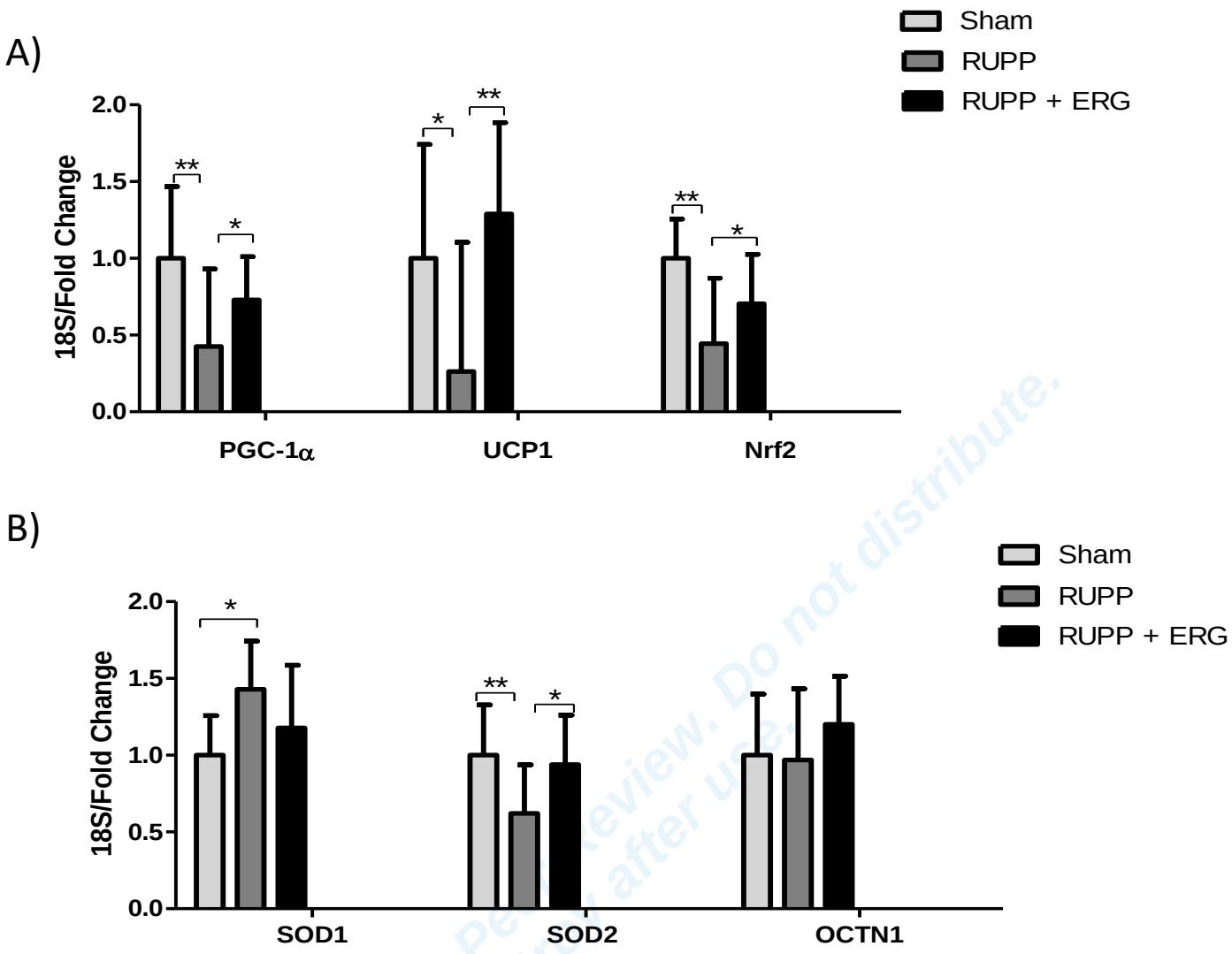

Figure 5: L-ergothioneine regulates placental expression of mitochondrial-ROS detoxifying enzymes in RUPP rats: A) Placental expression of PGC-1α, UCP-1 and Nrf2 expression were significantly reduced in RUPP rats (n=8) compared with sham group (n=8). Administration of L-ergothioneine significantly increased placental expression of all these mitochondrial markers in RUPP rats. B) Placental expression of antioxidant SOD1 was significantly increased while mitochondrial SOD2 expression was significantly decreased in the RUPP group compared to sham group. Administration of L-ergothioneine significantly increased mitochondrial antioxidant SOD2 expression in RUPP rats. OCTN1 expression was not significantly different between any of the studied groups. Data are presented as mean ± SEM, *P<0.05, **P<0.01.

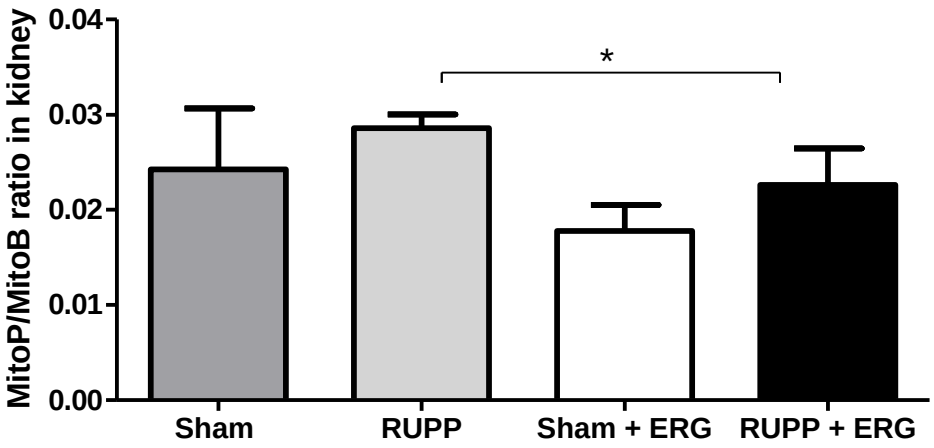

Figure 6: L-ergothioneine reduces mitochondrial-specific H_2O_2 levels.

Rats were injected with MitoB for 4 hrs and the ratios of MitoP/MitoB were determined by LC/MS/MS. Data is represented as Mean ± SEM of 3 samples per group and corrected for ratio. Statistical analysis was determined by a two-tailed student t-test: * $P < 0.05$.

Title: L-(+)-ergothioneine significantly improves the clinical characteristics of preeclampsia in the reduced uterine perfusion pressure rat model

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Short Title: L-ergothioneine as a treatment for Preeclampsia

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Abstract

Preeclampsia is a multifactorial hypertensive disorder of pregnancy founded on abnormal placentation, and the resultant placental ischemic microenvironment is thought to play a crucial role in its pathophysiology. Placental ischemia due to fluctuations in the delivery of oxygen results in oxidative stress, and recent evidence suggests that mitochondrial dysfunction may be a prime mediator. However, large clinical trials of therapeutic antioxidants such as vitamins C and E for the treatment of preeclampsia have been disappointing. L-(+)-ergothioneine (ERG), an unusual amino acid betaine derived from histidine, has important cytoprotective and antioxidant properties under conditions of high oxidative stress. In this study, we investigated the potential therapeutic effects of administration of ERG in the reduced uterine perfusion pressure (RUPP) rat model of preeclampsia.

L-ergothioneine (25mg/kg/day) was administered to rats on gestational day 11. On gestational day 14 RUPP surgery was performed and on gestational day 19, blood pressure (mean arterial pressure) and fetal growth were measured. Production of mitochondrial-specific H_2O_2 was analysed *in vivo* in kidney samples. ERG ameliorated the hypertension and significantly increased pup weight in RUPP rats. ERG also significantly decreased circulating levels of anti-angiogenic sFlt-1 in RUPP rats. Mitochondrial-specific H_2O_2 was also significantly decreased in kidney tissue in RUPP rats treated with ERG. These data support the potential use of ERG for the treatment of preeclampsia.

Introduction

Preeclampsia is a multisystemic disorder of pregnancy which results in high blood pressure accompanied by and/or proteinuria, acute kidney injury (AKI) and/or liver dysfunction fetal growth restriction (FGR) after 20 weeks' gestation (1) It affects more than eight million pregnancies worldwide annually, and is the leading cause of maternal death (2). Despite extensive research, the exact pathophysiological mechanisms underlying this syndrome remain poorly elucidated. Nonetheless, defective placentation is strongly considered to be a critical event in the pathology of the disorder (2). Failure to remodel spiral arteries results in high-pressure blood-flow-mediated placental damage and intermittent fluctuations in oxygen delivery, which exposes the placenta to oxidative stress (3). The resultant placental ischaemic microenvironment is inherently linked to increased production and secretion of deleterious soluble mediators that provoke extensive maternal inflammation and endothelial dysfunction (4). Several reports have observed higher levels of markers of oxidative stress (including F2-isoprostanes, nitrotyrosine and 4-hydroxynonenal staining) in placental tissue from preeclamptic pregnancies compared with those from uncomplicated pregnancies (5-8).

While there are a number of different cellular sources of reactive oxygen species (ROS), mitochondria are the major cellular producers (9). Furthermore, in terms of preeclampsia, there is growing evidence incriminating mitochondrial dysfunction in its underlying pathophysiology (10). Initial studies showed increased mitochondrial lipid peroxidation and enhanced susceptibility to oxidative damage in placental tissue of pregnancies complicated by preeclampsia (11). More recently, work has confirmed this association with strong evidence of perturbation of mitochondrial

function in the metabolite profile of plasma samples taken at 15 week's gestation from patients who subsequently developed preeclampsia (12).

While there is significant evidence for the pathogenic role of oxidative stress in the development of preeclampsia, clinical trials of antioxidant interventions were disappointing and not clinically effective in treating the disorder. One very plausible explanation is that these antioxidants missed the intracellular location of ROS production, namely the mitochondria; hence they have failed to alleviate the pathological oxidative damage. Another is that molecules such as L-ascorbate can actually be pro-oxidant in the presence of free iron (9). L-ergothioneine (ERG) is an unusual thio-histidine betaine amino acid and is a naturally occurring anti-oxidant discovered over a century ago in the rye ergot (13-16). The predominant role of ERG, via a variety of mechanisms, is to serve as an antioxidant and cellular protectant against various kinds of reactive oxygen species (17). Additionally, there has been some circumstantial evidence that ERG could target mitochondria (18) and hence could dampen exaggerated mitochondrial-specific ROS in response to oxidative stress.

Therefore, we aimed to investigate the role of ERG as a potential therapeutic target for preeclampsia using the reduced uterine perfusion pressure (RUPP) model in pregnant rats. The placental ischaemic RUPP model has numerous features of preeclampsia that are clinically evident in women and has been used as a preclinical model for the investigation of novel therapeutic targets for the treatment of preeclampsia (19, 20). Moreover, we additionally wanted to examine if ERG ameliorated the clinical characteristics of preeclampsia in part via regulation of mitochondrial-specific ROS production. To this end, we used a novel ratiometric mass spectrometry probe MitoB that specifically accumulates in mitochondria and

generates a MitoP phenol product upon reaction with H_2O_2 which can be subsequently be analysed *ex vivo* by mass spectrometry.

Materials and Methods

Animals:

Sprague Dawley-timed pregnant rats were supplied and maintained by the University College Cork Biological Services Unit. Animals were maintained at a temperature of 21˚C, with a 12-hour light/dark cycle and free access to food and tap water. All the procedures were performed in accordance with National Guidelines and the European Directive 2010/63/EU, under an authorization issued by the Health Products Regulatory Authority Ireland and approved by the Animal Ethics Committee of University College Cork (AE19130/P037).

RUPP procedure

The RUPP procedure is a well-established surgical model for studying the link between placental ischemia and hypertension in the pregnant rat and has been previously described in detail (19, 21). In brief, on gestational day (GD) 14, under isoflurane anaesthesia, RUPP reduction in blood flow to the uteroplacental unit was achieved by placing a silver clip (0.2 mm ID) on the abdominal aorta (one clip) above the iliac bifurcation. Two further clips (0.1 mm ID) were carefully placed around the left and right ovarian arteries. Sham surgery was performed as controls, which involved abdominal incision but didn't involve insertion of any clips on either abdominal aorta or ovarian arteries. On GD18, a chronic indwelling catheter was

inserted into the carotid artery and on GD19 mean arterial blood pressure (MABP) was recorded in conscious animals.

L-ergothioneine *in vivo* experimental protocol

Four experimental groups were used to investigate the effect of administration of ERG in the RUPP rat model of pre-eclampsia. Pregnant rats were divided into Sham (n=8), Sham+ERG (n=8) or RUPP (n=8), and RUPP+ERG (n=8). ERG was administered at (25mg/kg/day) in their drinking water on GD11 until the end of the experiment on GD19. The dose for ERG (25mg/kg/day) were selected based on previously published rodent studies using this antioxidant (22, 23). L-ergothioneine was provided by Tetrahedron (Paris, France; www.tetrahedron.fr).

Plasma Collection

Blood collected from EDTA vacutainers were centrifuged at 2000 x g and 2400 x g for 10 minutes at 4˚C, plasma was removed and stored at -80˚C for further analysis.

Urine analysis

On GD18, each rat was singularly housed in a metabolic cage and urine collected overnight. All samples were stored immediately following collection at -80ºC. Microalbumin:creatinine ratios were calculated following measurement of albumin using an immuno-turbidimetric test for the quantitative determination of albumin in an OLYMPUS AU5832 analyser® and urine creatinine using a kinetic colour test (Jaffé method) (24). Similarly, protein: creatinine ratios were calculated following measurement of protein by adding benzethonium chloride which resulted in the formation of a fine suspension, which was then quantified turbidimetrically at 525 nm using an OLYMPUS analyser®.

Measurement of anti-angiogenic protein sFlt-1 by ELISA

Circulating sFlt-1 concentration in plasma samples from all experimental groups were quantified by enzyme-linked immunosorbent assay (ELISA) using a Quantikine sFlt-1 immunoassay (R&D Systems) as per manufacturers' instructions.

Isolation of RNA and Real-time PCR analysis

RNA was extracted from placental tissue using the Trizol method. SLC22A4 (ERG transporter), superoxide dismutase 1 (SOD1), SOD2, and uncoupling protein-1 (UCP-1), PCG-1α and Nrf2 gene expression was quantified by Real-time PCR using StepONE Plus Detection system. Taqman assays (Applied Biosciences) and Sybr Green primers were used for quantification. The amounts of the target genes were normalised to the geometric mean of internal control gene 18S, and were determined using the comparative $2^{-\Delta\Delta CT}$ method.

Isometric Myography

In all groups, third order mesenteric arteries were dissected and mounted on a 4-channel wire myograph (Model 610 mol/L Danish Myo Technology) containing oxygenated (95% O_2 and 5% CO_2) physiological salt solution (PSS) at 37˚C. Vessels were normalized to achieve a transmural pressure of 100mmHg using the DMT Normalization software. Isometric tension was recorded and displayed using Powerlab Chart Software (AD Instruments). The viability of the smooth muscle was examined by the addition of a 123 mM KCl solution. After PSS washes, concentration responses were carried out with thromboxane mimetic U46619 (9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$; 10^{-9} to 10^{-5} M) ,and either

Bradykinin (BK; 10^{-8} to 10^{-5} M), Acetylcholine (ACh; 10^{-8} to 10^{-5} M), or Sodium Nitroprusside (SNP; 10^{-8} to 10^{-5} M) respectively (25, 26).

Estimation of mitochondrial H_2O_2 in the RUPP model *in vivo*

Mitochondrial hydrogen peroxide was measured *in vivo* using the Cayman Chemical Hydrogen Peroxide Ratiometric MaxSpec kit based on the MitoB mass spectrometric probe method described previously (27). Briefly, 75 nmol MitoB in 50µl saline was administered by tail-vein injection to rats in the 4 experimental groups on GD19, 4 hours prior to end of the experiment. At the end of the procedure, kidney tissues were dissected out, snap frozen and stored at −80 °C. For mitochondrial H_2O_2 analysis, kidney tissues were homogenised, spiked with deuterated internal standards and MitoB and its product MitoP were extracted using acetonitrile/formic acid. MitoB and MitoP present in kidney tissue were measured using UPLC Xevo TQD mass spectrometer (Waters) and the amounts of MitoP and MitoB in each sample were determined relative to a standard curve. The MitoP/MitoB ratios for each sample were then calculated.

Statistical analysis

All data are expressed as mean ± SEM, or fold change relative to control. Analysis was performed using GraphPad Prism and student *t* test was applied when comparisons were made between 2 groups.

Results

L-ergothioneine ameliorated RUPP-induced hypertension

Mean arterial blood pressure (MABP) was significantly increased in the RUPP group compared with sham group (129 ± 3 mmHg vs 117 ± 7 mmHg, P = 0.05; N=8, Figure 1A). Furthermore, administration of ERG significantly reduced MABP in the RUPP rats (129 ± 3 mmHg v 115 ± 4 mmHg), P = 0.01; N=8, Figure 1A). MABP in sham rats treated with ERG was not significantly different from RUPP rats treated with ERG, indicating the beneficial effect of ERG antioxidant in reducing blood pressure occurs in response to placental ischemia. There was no significant difference in microalbumin:creatinine (MCR) ratio in the RUPP group compared to Sham group, nor was there a significant difference in the MCR ratio in the RUPP rats treated with ERG (Figure 1B).

L-ergothioneine improves fetal weight

Fetal birth weight was significantly decreased in the RUPP group compared to the Sham group (1.8 ± 0.04g v 2 ± 0.03g; P= 0.0004; Figure 2A). Administration of ERG significantly rescued fetal growth restriction in the RUPP rats (2 ± 0.03 g v 1.8 ± 0.04g, P= 0.0006; Figure 2A). Placental weights were significantly reduced in RUPP group compared to Sham group (0.4 ± 0.01g v 0.5 ± 0.01g; P= 0.002, Figure 2B). The administration of ERG had no effect on placental weight in the RUPP rats (0.4 ± 0.01g v 0.4 ± 0.01g) (Figure 2B). A significant decrease in pup number was observed in the RUPP group compared to Sham group (11 ± 1 v 14 ± 1 P= 0.02, Figure 3C). There was no significant difference in pup number in RUPP rats treated with ERG relative to RUPP rats (13 ± 2 v 11 ± 1). Pup weight was not significantly different between Sham or RUPP rats treated with ERG, indicating the beneficial

effect of ERG in rescuing fetal weight during placental ischemia. There was no significant difference in the crown to rump length (CRL) of pups in any of the studied groups (Figure S1. Supplemental Material). Finally, maternal weight did not differ between any of the studied groups. (Figure S2. Supplemental Material).

L-ergothioneine reduces circulating sFlt-1 levels

There was a significant increase in the circulating soluble anti-angiogenic mediator sFlt-1 in the RUPP group compared to the sham group (1995 ± 97 pg/ml v 1185± 349 pg/ml, P= 0.04) (Figure 3). Administration of ERG significantly decreased circulating sFlt-1 levels in RUPP rats (1367 ± 245 pg/ml, P= 0.04; Figure 3).

Effect of L-ergothioneine on vasorelaxation in the RUPP model

Mesenteric arteries from the RUPP group displayed impaired vasorelaxation in response to bradykinin (BK) when compared to the sham group (R_{max}: 28 ± 7% versus 52 ± 9%; P= 0.01, log EC50: -6.6 ± 0.2 versus -6.8 ± 0.3 mol/L; P=0.7, Figure 4A). However, no significant differences were seen in the vascular response to the endothelial independent vasodilator sodium nitroprusside (SNP) (R_{max}: 78 ± 5% versus 80 ± 5%; P= 0.36, log EC50: -7.1 ± 0.2 versus -7.2 ± 0.4 mol/L, P=0.9, Figure S3.A) or Acetylcholine (ACh) (R_{max}: 83 ± 8% versus 81 ± 6%; P= 0.1, log EC50: -7.1 ± 0.2 versus -7 ± 0.1mol/L, P= 0.6 Figure S.3B). Treatment with ERG had no significant effect on the vasorelaxant responses of mesenteric vessels in response to BK (R_{max}: 23 ± 5% versus 28 ± 7%; P= 0.9, log EC50:-6.7 ± 0.2 versus -6.8 ± 0.2 mol/L, P=0.7, Figure 4B), or SNP (R_{max}: 78 ± 6% versus 74 ± 4%; P= 0.8, log EC50: -7.1 ± 0.2 versus -7.2 ± 0.2, P=0.9, Figure 4.SA and B) in RUPP rats.

L-ergothioneine alters placental expression of markers of mitochondrial ROS-detoxifying enzymes

Placental expression of mitochondrial orchestrator proliferator activated receptor γ co-activator 1-α (PGC-1α) (1 ± 0.4 fold vs 0.4± 0.5 fold, n=8, P= 0.01, Figure 5A) and mitochondrial-ROS detoxifying enzymes including uncoupling protein 1 (UCP-1) (1 ± 0.5 fold vs 0.2 ± 0.5 fold, P= 0.04) were significantly decreased in the RUPP rats. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a transcriptional regulator of the mitochondrial antioxidant defence system and coactivated by PGC-1α was also significantly reduced in the RUPP rats (1 ± 0.2 fold vs 0.4 ± 0.4 fold, n=8, P= 0.01, Figure 5A). Placental expression of SOD1 antioxidant was significantly increased (1 ± 0.3 fold vs 1.4 ± 0.3 fold, n=8, P= 0.02; Figure 5B), while mitochondrial SOD2 antioxidant was significantly reduced (1 ± 0.3 fold vs 0.6 ± 0.3 fold, n=8, P= 0.01, Figure 5B) in RUPP rats compared with sham group.

ERG treatment significantly increased placental expression of PGC-1α (1.7 ± 0.3 fold vs 1 ± 0.5 fold, n=8, P= 0.02), UCP-1 (4.9 ± 0.6 fold vs 1 ± 0.5 fold, n=8, P= 0.001) and Nrf2 (1.6 ± 0.3 fold vs 1 ± 0.4 fold, n=8, P= 0.02, Figure 5A) in RUPP rats. ERG treatment significantly increased placental expression of SOD2 in RUPP rats (1.5 ± 0.3 fold vs 1 ± 0.3 fold, n=8, P= 0.02, Figure 5B). There was no significant difference in placental expression of the ERG transporter OCTN1 in any of the experimental groups studied (Figure 5B).

L-ergothioneine reduces mitochondria-specific H_2O_2 production in the kidney

MitoP/MitoB ratio was increased in kidney tissue of RUPP rats compared with the sham group (0.029 ± 0.001 v 0.024 ± 0.006; n=3, P=0.3, Figure 6), indicating for the first time that mitochondrial-specific H_2O_2 is increased *in vivo* as a result of placental ischaemia. Furthermore, pre-treatment with ERG significantly reduced the MitoP/MitoB ratio in kidney tissue in RUPP rats (0.022 ± 0.003 v 0.029 ± 0.001; n=3, P=0.05, Figure 6), establishing that L-ergothioneine reduces mitochondrial-specific H_2O_2 production *in vivo.*

Discussion

Despite a significant amount of evidence for the pathological role of oxidative stress in the development of preeclampsia, clinical data from two major antioxidant vitamin trials have been negative (28, 29). One possible reason for these negative findings may be due to the fact these interventions missed the primary intracellular producer of ROS, namely the mitochondria; or because in the presence of free or poorly liganded iron substances such as ascorbate are actually pro-oxidant (9). In the present study, L-ergothioneine, an amino acid with potent antioxidant properties, attenuated hypertension and rescued fetal growth restriction in the preclinical RUPP rat model of preeclampsia, which closely mimics many aspects of preeclampsia during human pregnancy. Additionally, this study also reported the novel finding of reduced mitochondrial H_2O_2 levels *in vivo* following L-ergothioneine administration. This work has highlighted that L-ergothioneine acts as a potent antioxidant, that ameliorates a number of phenotypic features of preeclampsia in a preclinical model of disease and mediated in part by the reduction of mitochondrial-specific ROS. This work consequently proposes the potential of ERG as a viable therapeutic for the prevention of preeclampsia.

L-ergothioneine is a water-soluble amino acid that is derived entirely from dietary sources. It has garnered much attention recently as a potential therapeutic intervention (13, 30-33), partly due to its preferential accumulation within tissues undergoing significant oxidative stress. A recent study investigating the reproductive safety profile of ERG in pregnant Sprague Dawley rats, established that ERG was well tolerated and with no adverse effects on a number of parameters (number of mating days, gestation length, pup viability index or litter parameters) (34). Furthermore, ERG treatment of diabetic rats significantly improved embryo

formation and quality (31). Collectively these data suggest ERG treatment may be safe to use in pregnancy and may prevent embryo malformations mediated by oxidative stress early in pregnancy. Consequently, we decided to administer ERG at an early point in pregnancy (GD11) in the preclinical RUPP model of preeclampsia.

The therapeutic effects of ERG in response to induced tissue damage in models of ischemia-reperfusion injury in the liver (35) and intestine (22) have previously been reported by the dampening of markers of oxidative stress and inflammation. Our novel data using ERG reported a reduction in hypertension and an improvement in fetal weight in response to placental ischemia in the preclinical RUPP model of preeclampsia, in part due to regulation of mitochondrial-specific ROS. Recent evidence identified a prominent role for mitochondrial-specific ROS (mROS) in modulating hypertension (36)**.** Using two *in vivo* murine models of hypertension (Ang II-induced and DOCA salt), this group established that using a mitochondria-targeted antioxidant (Mito-Tempo) alleviated endothelial dysfunction, reduced vascular mitochondrial superoxide and subsequent hypertension (36). Furthermore, similar to our work, Vaka et al showed in the preclinical RUPP model that placental ischemia dysregulated mitochondrial function with elevated mROS and identified that treatment with mitochondrial targeted antioxidants attenuated hypertension with improvement in fetal outcomes in treated RUPP rats (37).

Deleterious circulating mediators including sFlt-1 are secreted in response to placental ischemia and have devastating consequences on the maternal vasculature. Furthermore, we previously detected increased levels of mitochondrial-specific superoxide production in HUVEC's incubated with plasma from women with preeclampsia compared with matched controls and non-pregnant controls (38). Additionally, Zsengeller et al. established an inverse correlation between placental

sFlt-1 and mitochondrial complex IV, suggesting that sFlt-1 may be harmful to mitochondria in preeclampsia (30). In our study, ERG treatment reduced the circulating levels of sFlt-1 in RUPP rats, indicating that ERG may preserve mitochondrial function in part by reducing the availability of sFlt-1 to induce mitochondrial damage. The reduction in sFlt-1 as a result of ERG treatment did not result in a subsequent reduction in maternal vascular dysfunction in the RUPP rats.

The capacity to accurately measure the concentration of reactive oxygen species, in particular mitochondrial-specific ROS *in vivo*, has proved to be extremely challenging yet is essential in understanding their physiological roles in certain diseases. The detection of mitochondrial-specific ROS such as superoxide using fluorescent probes can be inferred, with certain caveats, by determining the changes in fluorescence, or ex-vivo tissue measurements of ETC complexes but these methods can be less selective and sensitive. One potential alternative is to use exogenous ratiometric probes (MitoB) injected into the animal models, that readily accumulate in mitochondria of tissues due to its possession of a triphenylphosphonium cation and which reacts with H_2O_2 *in vivo* to produce a diagnostic exomarker (MitoP) that can be accurately quantified by mass spectrometry (39).

In this study, we have shown for the first time that this method of measuring mitochondrial H_2O_2 production *in vivo* is applicable in a rat model, without any adverse effects on reproductive safety. Furthermore, we reported that mitochondrial H_2O_2 was increased in kidney tissue in the RUPP group compared with the sham group. This is in agreement with recent work by Vaka et al, who showed an increase in mROS in kidney tissue in the RUPP group by examining ETC activity and cellular respiration *ex vivo* using isolated mitochondria from kidney tissues (37).

More importantly, treatment with ERG resulted in a significant reduction in mitochondrial-specific H_2O_2 production in kidney tissue in RUPP rats, indicating that ERG may be mediating its therapeutic effects in part by directly reducing mROS production in the preclinical model of preeclampsia. Further evidence to support our theory is provided by the fact that placental expression of markers of mitochondrial-ROS detoxifying enzymes and their transcriptional regulators were significantly improved in ERG-treated RUPP rats, in contrast to their reduced expression in RUPP rats.

At present, only one study has investigated the pharmacokinetics of ERG administration in human subjects (30). ERG was rapidly absorbed and retained within the tissue/plasma with relatively low urinary excretion (<4% of administered dose). This work was further extended by Tang et al. who established that ERG and its metabolites are widely distributed in various tissues in male mice administered ERG over a range of days. This group also established that ERG was highly retained in the body and suggested this could be as a result of possible reabsorption by the kidneys (40).

This study provides evidence of the therapeutic potential of ERG in a preclinical model of preeclampsia. ERG improved both hypertension and fetal weight in the RUPP rat model of preeclampsia. Furthermore, ERG treatment significantly altered mitochondrial function in both the kidney and placental tissue which may in part be responsible for the beneficial effects on phenotypic features of preeclampsia in the RUPP model. Given its favourable safety profile, its long half-life and resistance to auto-oxidation and multiple mechanisms of action, not least its ability to regulate mitochondrial function, further studies are needed to explicitly define the protective mechanisms of ERG in treating preeclampsia in humans.

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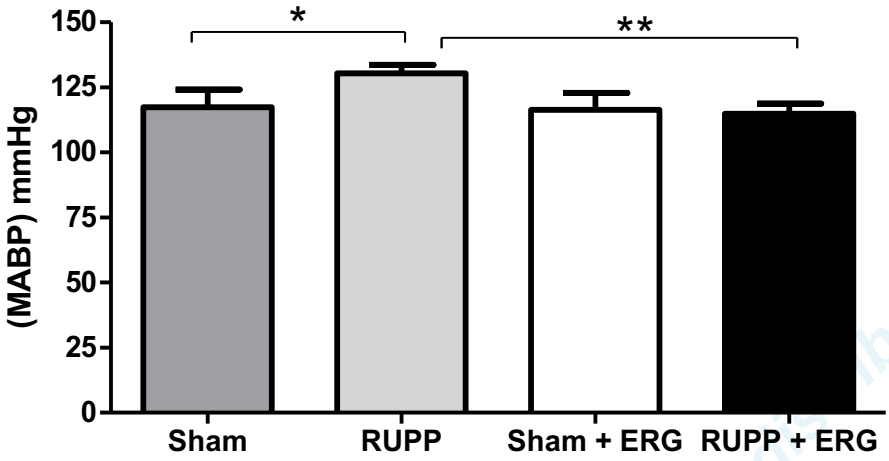

Figure 1A: L-ergothioneine attenuates blood pressure in reduced uterine perfusion pressure (RUPP) rats: a) RUPP rats (n=8) showed significantly elevated mean arterial blood pressure (MABP) compared with sham group (n=8). L-ergothioneine (25mg/kg/day) reduced MABP in RUPP rats (n=8). Data is presented as mean ± SEM, *P<0.05, **P<0.01.

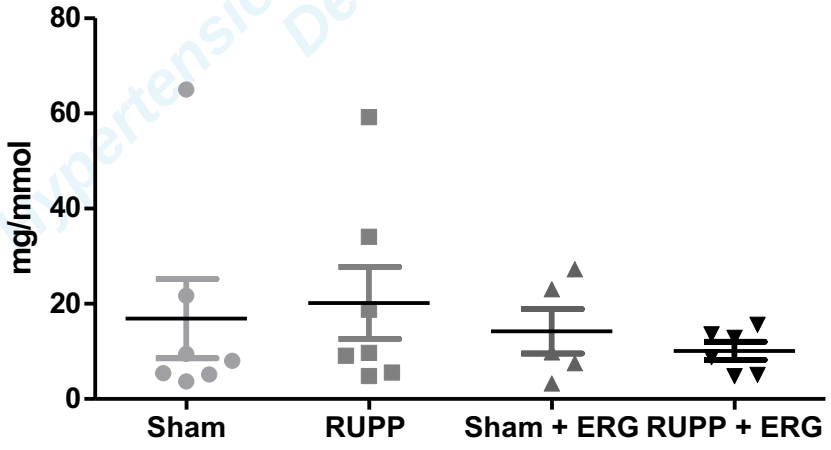

Figure 1B: L-ergothioneine did not alter microalbumin:creatinine ratio in RUPP rats: Administration of L-ergothioneine (25mg/kg/day) did not significantly affect microalbumin:creatinine ratio in RUPP rats.

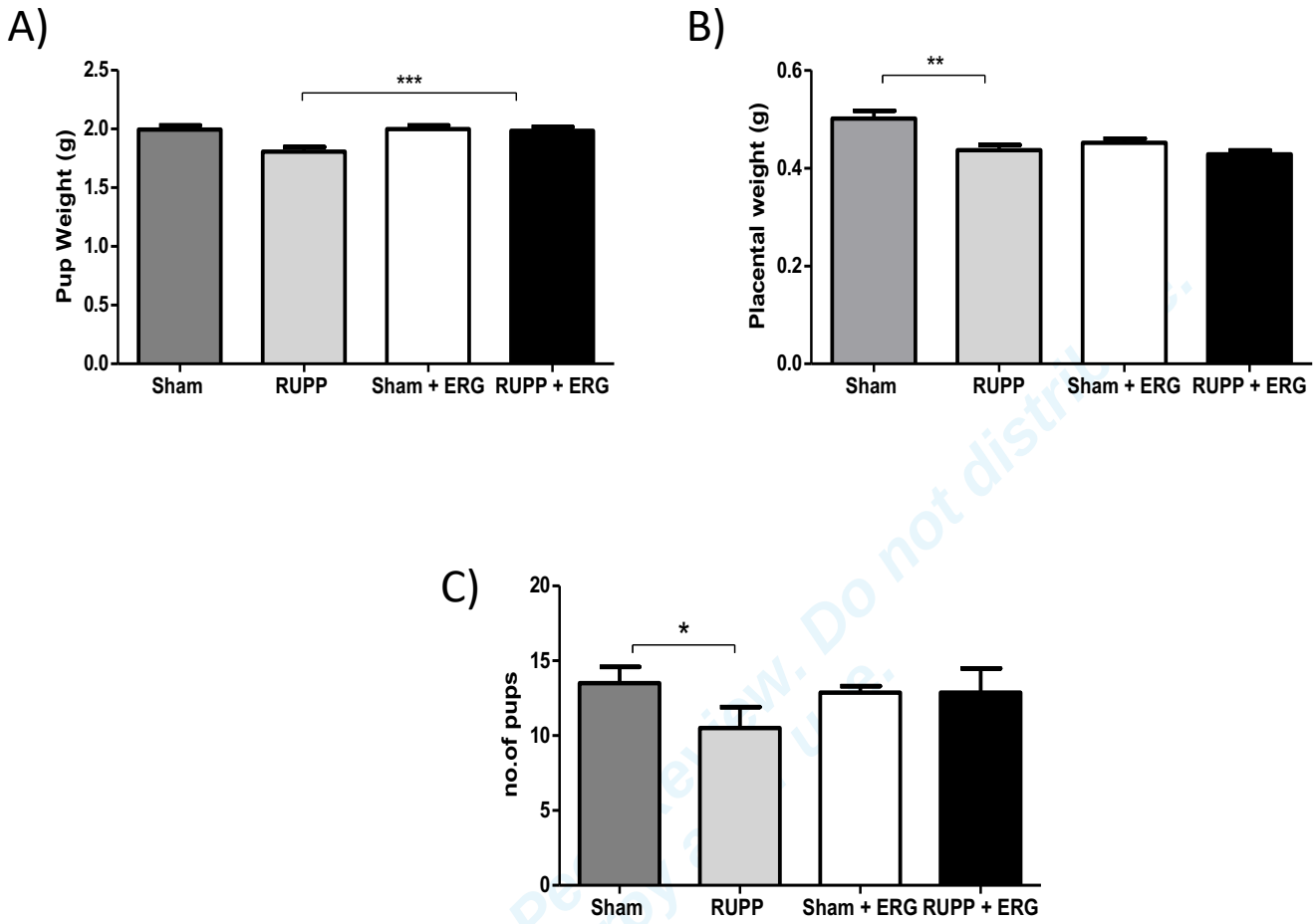

Figure 2: L-ergothioneine improves fetal outcomes in RUPP rats: RUPP rats (n=8) show reduced A) pup weight and B) placental weight and C) litter size compared with sham group (n=8). Administration of L-ergothioneine (25mg/kg/day) improved fetal outcome in RUPP rats. Data are presented as mean ± SEM, *P<0.05, **P<0.01, ***P<0.001.

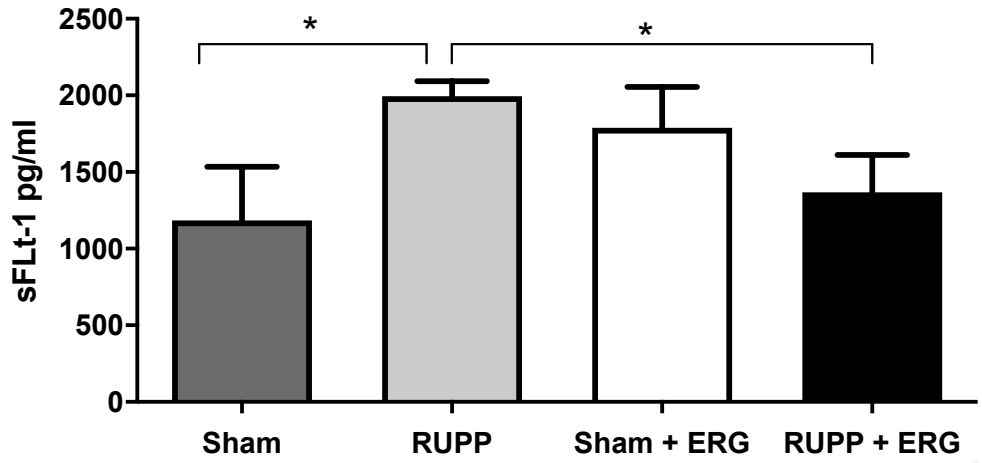

Figure 3: L-ergothioneine reduces circulating anti-angiogenic factor sFlt-1 in RUPP rats: Circulating levels of sFlt-1 were significantly increased in RUPP rats (n=8) compared with sham controls (n=8). Administration of L-ergothioneine significantly reduced circulating sFlt-1 levels in RUPP rats. Data is presented as mean ± SEM, *P<0.05.

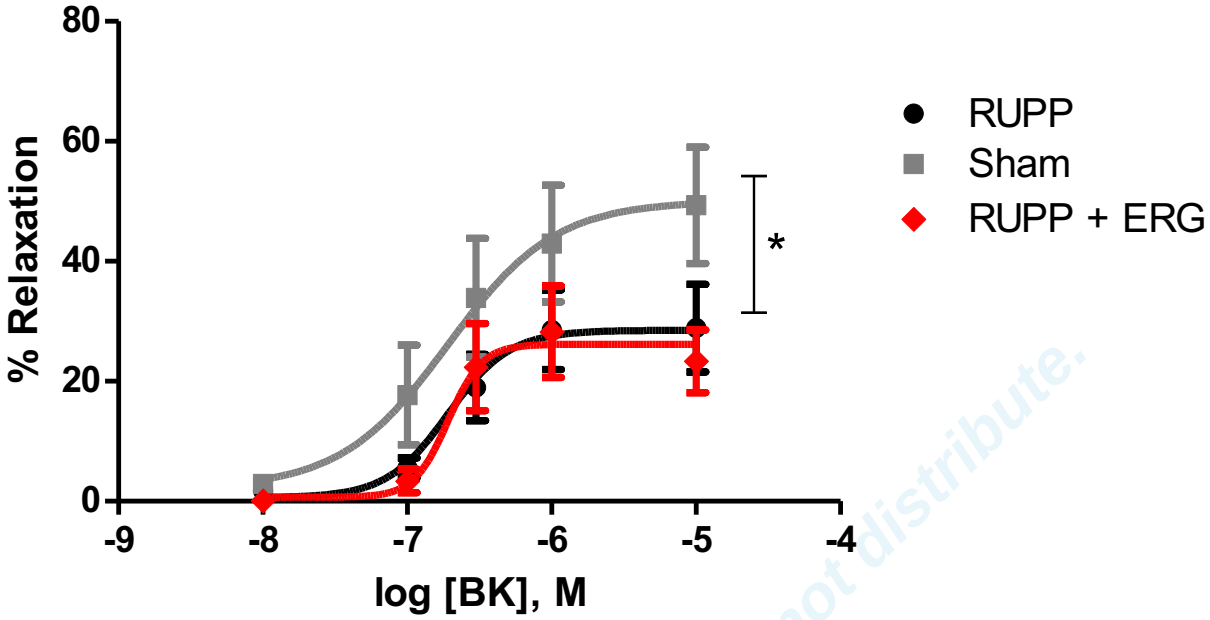

Figure 4: Impaired vasorelaxtion in mesenteric arteries in RUPP rats: RUPP rats (n=8) showed significantly impaired vasorelaxation in response to bradykinin compared to the sham group (n=8). Administration of L-ergothioneine did not attenuate the impaired vasorelaxation to bradykinin. Relaxation is calculated as a percentage of the maximum contraction and expressed as mean ± SEM. *P<0.05.

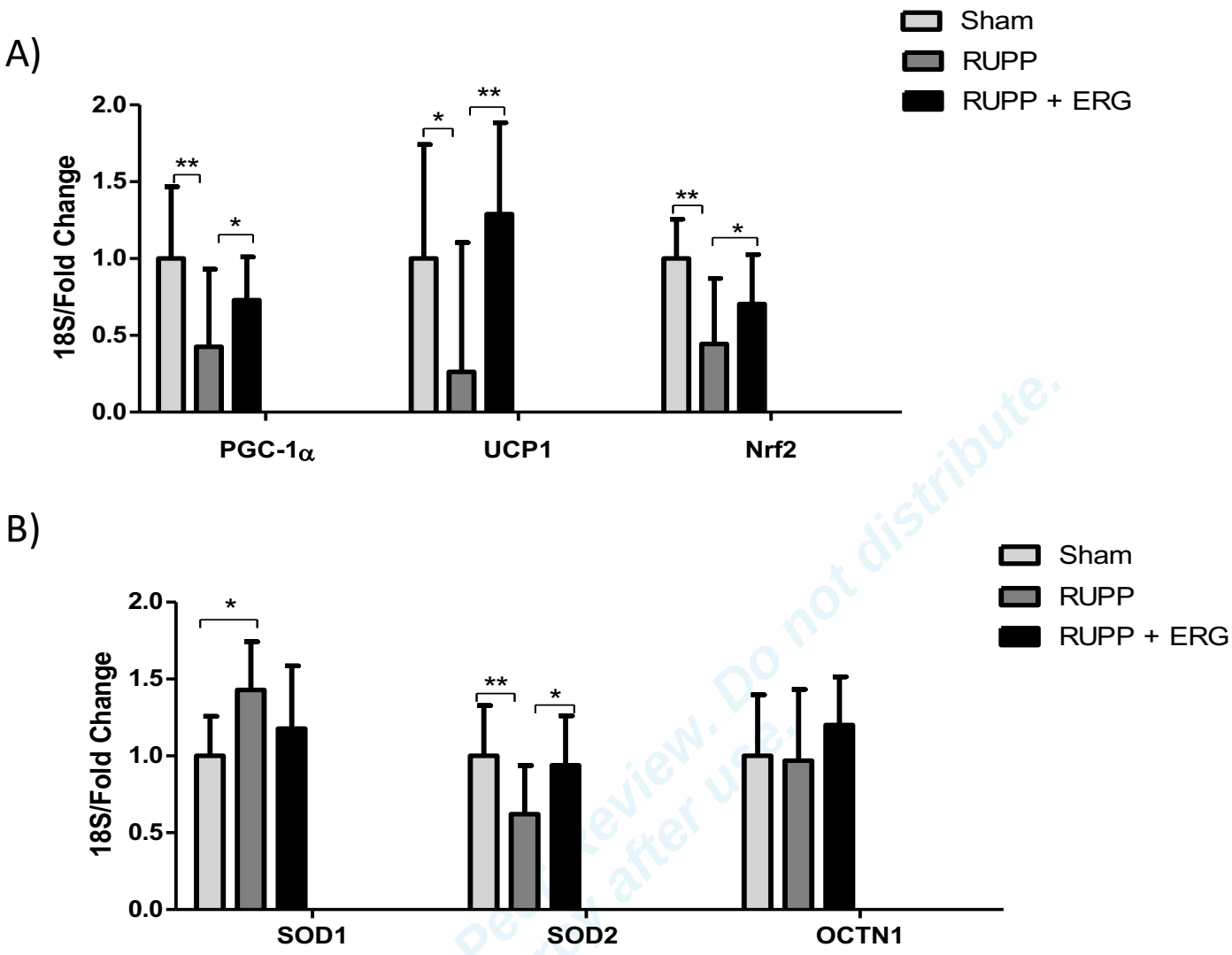

Figure 5: L-ergothioneine regulates placental expression of mitochondrial-ROS detoxifying enzymes in RUPP rats: A) Placental expression of PGC-1α, UCP-1 and Nrf2 expression were significantly reduced in RUPP rats (n=8) compared with sham group (n=8). Administration of L-ergothioneine significantly increased placental expression of all these mitochondrial markers in RUPP rats. B) Placental expression of antioxidant SOD1 was significantly increased while mitochondrial SOD2 expression was significantly decreased in the RUPP group compared to sham group. Administration of L-ergothioneine significantly increased mitochondrial antioxidant SOD2 expression in RUPP rats. OCTN1 expression was not significantly different between any of the studied groups. Data are presented as mean ± SEM, *P<0.05, **P<0.01.

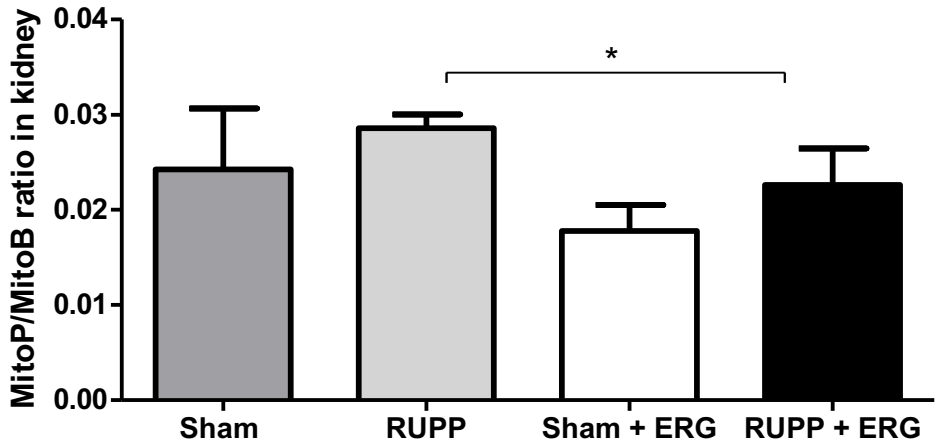

Figure 6: L-ergothioneine reduces mitochondrial-specific H_2O_2 levels.

Rats were injected with MitoB for 4 hrs and the ratios of MitoP/MitoB were determined by LC/MS/MS. Data is represented as Mean ± SEM of 3 samples per group and corrected for ratio. Statistical analysis was determined by a two-tailed student t-test: * $P < 0.05$.